REMARKS

Reconsideration of the Office Action mailed June 26, 2003, (hereinafter "instant Office Action"), entry of the foregoing amendments, withdrawal of the rejection of claims 21-27, 32 and 33 and the withdrawal of the objection to claims 21-24, 26 and 27 are respectfully requested.

In the instant Office Action, the Examiner has made the Restriction Requirement final. Claims 1-88 are listed as pending, claims 1-20, 28-31 and 34-88 are withdrawn from consideration, claims 21-27, 32 and 33 are listed as rejected and claims 21-24, 26 and 27 are objected to.

The Examiner has objected to the specification alleging that it contains "an embedded hyperlink and/or other form of browser-executable code", such as on page 23, line 14 and elsewhere. Applicants have deleted the references to websites on page 23, lines 14 and 19 and page 24, lines 11-12 and submit herewith replacement paragraphs.

The Examiner has objected to the specification because of the minor informality of "anaology" being misspelled on page 14, line 28. Applicants have corrected this misspelling and submit herewith a replacement paragraph.

Thus, the objections to the specification are obviated and should be withdrawn.

The Examiner has objected to Claims 21-24, 26 and 27 because of minor informalities as noted on page 3 of the instant Office Action. Applicants have amended claims 21-24, 26 and 27 to correct the minor informalities as suggested by the Examiner. Therefore, the objections to Claims 21-24, 26 and 27 are obviated and should be withdrawn.

The Examiner has rejected claims 21-27, 32 and 33 under 35 U.S.C. §112, first paragraph, alleging that the specification, while being enabling for the atomic coordinates for residues 802-1124 of Tie-2 and Inhibitor III complex, does not reasonably provide enablement for the atomic coordinates of an unbound version of a Tie-2 polypeptide or atomic coordinates of the complete polypeptide of Tie-2 and Inhibitor III complex. The Examiner alleges that the invention as presently stated in claim 21 encompasses these additional sets of atomic coordinates, but that they are not included in the specification which consequently causes a lack of scope of enablement of the instant invention for one of ordinary skill in the art.

Specifically, the Examiner asserts that Applicants have not provided enablement for finding atomic coordinates of an unbound Tie-2 polypeptide as well as an entire Tie-2

polypeptide and Inhibitor III complex as encompassed in claim 21. Applicants respectfully traverse this rejection.

Applicants respectfully point out that Claim 21 is a method claim directed to identifying a compound that is an inhibitor of a Tie-2 protein. One step of Claim 21 is limited to obtaining the atomic coordinates of a crystal of a polypeptide comprising the *catalytic domain* of a Tie-2 protein. On page 10, lines 3-6, Applicants state "For Tie-2, the catalytic domain is defined by amino acid residues from about residue 828 to about residue 985 of SEQ ID NO: 1, with residues 828-840, 853-855, 872, 873, 876, 879, 880, 885-888, 900, 902-909, 912, 954, 955, 960, 964, 968-971 and 980-985 included in the catalytic domain." Therefore, the use of at least those coordinates comprising the *catalytic domain* of a Tie-2 protein is the defining feature of the instant invention and Applicants have enabled how to obtain and use them.

With respect to the Examiner's allegation that the instant specification is not enabling based on the amount of direction or guidance provided, Applicants submit that they have shown possession of the instant invention by reducing it to practice (although Applicants are not required to do so). Specifically, Applicants have shown how to solve the crystal structure of a polypeptide comprising the catalytic domain of a Tie-2 protein on page 48, line 28 to page 49, line 14 and page 50, lines 1-27 and how to define the active subsites on page 50, lines 10-19, using various computer programs. On page 51, lines 1-7 Applicants teach inhibitor docking. M.P.E.P 2163 further states that "An application specification may show actual reduction to practice by describing testing of the claimed invention or, in the case of biological materials, by specifically describing a deposit made in accordance with 37 CFR 1.1801 *et seq.*"

Applicants respectfully point out that there is no requirement as to how many working examples must be provided in a patent application. With respect to working examples, the Court of Customs and Patent Appeals had stated earlier in In re Cavallito and Gray, 282 F. 2d 363, 127 USPQ 206 (CCPA 1960), in deciding the issue of sufficiency of disclosure of the appellant's specification, that "....it is the nature of the disclosure rather than the number of examples given which determines the sufficiency of the disclosure...." In re Cavallito and Gray, supra 127 USPQ 208. In Example 2 Applicants have demonstrated identifying a compound which is an inhibitor of Tie-2 by obtaining the atomic coordinates of a crystal of a polypeptide comprising the catalytic domain of a Tie-2 protein, using these atomic coordinates to define the active subsites of Tie-2 and identifying a compound which binds to one or more active subsites and inhibit the Tie-2

protein. Applicants have shown sufficient examples to demonstrate that the instant method works.

Based upon the foregoing, the rejection of claims 21-27, 32 and 33 under 35 U.S.C. §112, first paragraph, is obviated and should be withdrawn.

The Examiner has rejected Claims 21-27, 32 and 33 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time of the invention was filed, had possession of the claimed invention. Applicants respectfully traverse this rejection.

The Examiner has confused the issue. 35 U.S.C. §112, first paragraph, as it relates to written description, is separate and distinct from the enablement requirement. *In re Barker*, 559 F.2d 588, 194, USPQ 470 (CCPA 1977), *cert. denied*, 434 U.S. 1064 (1978); *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1562, 19 USPQ2d 1111, 1115 (Fed. Cir. 1991). Adequate description under the first paragraph of 35 U.S.C. 112 does not require literal support for the claimed invention. *In* re *Herschler*, 591 F.2d 693, 200 USPQ [*5] 711 (CCPA 1979); *In re Edwards,* 568 F.2d 1349, 196 USPQ 465 (CCPA 1978); *In re Wertheim*, 541 F.2d 257, 191 USPQ 90 (CCPA 1976).

The Examiner cites *Fiers v. Revel*, 24 USPQ2d 1601, 1601 (CAFC 1993) and *Amgen, Inc. v. Chugai Pharmaceutical Co. Ltd.,* 18 USPQ2d 1016, stating "the skilled artisan cannot envision the detailed chemical structure of the encompassed polynucleotides and/or proteins, regardless of the complexity or simplicity of the method of isolation. Adequate written description requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it. The nucleic acid itself is required." With respect to *Fiers v. Revel*, Applicants respectfully point out that the claim being examined was directed to a DNA, whereas in the instant application the claim is directed to a method. In *Fiers* the applicant was claiming the DNA itself, so to require the applicant to provide the nucleic acid or the nucleotide sequence in order to prove possession of the invention is reasonable. In the instant application, however, Applicants are claiming a method. One step of this method is to obtain the atomic coordinates of a crystal of a polypeptide comprising the catalytic domain of a Tie-2 protein. The only requirements for Applicants to show that they have met the written description requirement, that is, to show possession of the invention, is to provide adequate disclosure to allow another to

practice the invention. Unlike *Fiers*, Applicants have done this by providing examples that illustrate the invention.

With respect to *Amgen, Inc. v. Chugai Pharmaceutical Co. Ltd., 18 USPQ2d 1016,* the case involved production of EPO and whether the defendant had disclosed the best mode and had enabled the claims. There was no question as to whether the defendant had provided adequate written description. The only reference to satisfying the written description requirement is in regard to patent applicants placing microorganism samples in a public depository when such a sample is necessary to carry out the claimed invention. Such a deposit is considered to satisfy the *enablement* requirement of 35 U.S.C. §112, when a written description alone would not place the invention in the hands of the public and physical possession of a unique biological material is required. Neither of these issues is relevant to the question of whether Applicants have satisfied the written description requirement in the instant application. In fact, in *Amgen* the court states: "...it is not necessary that a patent applicant test all the embodiments of his invention, *In re Angstadt*, 537 F.2d 498, 502, 190 U.S.P.W. (BNA) 214, 218 (CCPA 1976); what is necessary is that he provide a disclosure sufficient to enable one skilled in the art to carry out the invention commensurate with the scope of his claims." In the instant application Applicants have provided sufficient disclosure to allow one of ordinary skill in the art to practice the claimed invention.

The Examiner also cites *Fiddes v. Baird*, 30 USPQ2d 1481, 1483 wherein claims directed to mammalian FGF's were found unpatentable due to lack of written description for the broad class, as the specification provided only the bovine sequence. In *Fiddes*, the subject matter at issue was a recombinant DNA molecule, whereas in the instant application the claims are directed to a method. As discussed above with regard to *Fiers*, adequate written description of DNA requires a precise definition, such as by structure, formula, chemical name or physical properties. One way to satisfy the written description is to provide the sequence of the DNA. However, when claiming a method, as Applicants do in the claims under rejection, it is only necessary to provide adequate description of the steps of the method to show possession of the invention, which Applicants have done through description and real working examples. And to fulfill the written description requirement, a patent specification must describe an invention and do so in sufficient detail that one skilled in the art can clearly conclude that "the inventor invented the claimed invention" *Lockwood v. American Airlines, Inc.* 107 F.3d 1565, 1572, 41 USPQ2D (BNA) 1961, 1966 (1997).

Lastly the Examiner cites *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398, 1404, 1405. The claims at issue in that case are specific to a microorganism containing a human insulin cDNA, cDNA encoding vertebrate insulin and cDNA encoding mammalian insulin. Each of these claims is directed to cDNA whereas Applicants' claims are directed to a method. In *University of California,* the court used the same rationale as in *Fiers, Amgen* and *Fiddes.* That is, that DNA or cDNA, like a chemical entity, must be described with specificity such as structure, formula or sequence. In the instant application Applicants do not claim DNA or cDNA but a method of identifying inhibitors of Tie-2. That one step of the method requires obtaining the atomic coordinates of a polypeptide comprising the catalytic domain of a Tie-2 protein is not the same thing as claiming DNA. Applicants have defined the catalytic domain as discussed above. Through detailed description and working examples Applicants have described their invention in adequate detail and thus met the requirement for written description.

Written description is not required for the entire scope of the claims, only the invention. There is no obligation for the patentee to describe every possible embodiment of the invention at issue. In *Utter v. Hiraga*, 845 F.2d 993, 998, 6 U.S.P.Q.2d (BNA) 1709, 1714 (Fed. Cir. 1988), the court found "A specification may, within the meaning of 35 U.S.C. §112, paragraph 1, contain a written description of a broadly claimed invention without describing all species that claim encompasses". The Examiner cites *Amgen* to support her position that the written description is inadequate, but in *Amgen*, the court found that over 3,600 different EPO analogs could be made by substituting at only a single amino acid position, and over a million different analogs can be made by substituting three amino acids. In the instant case, the method is limited to the atomic coordinates of a polypeptide comprising the catalytic domain of a Tie-2 protein. This limits the polypeptides which can be used, since the catalytic domain of a Tie-2 protein must be present. Rather, it is sufficient if the originally-filed disclosure would have conveyed to one having ordinary skill in the art that an applicant had possession of the concept of what is claimed. *In re Anderson*, 471 F.2d 1237, 176 USPQ 331 (CCPA 1973).

With respect to satisfying the written description requirement, Chisum on Patents states the following:

> To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. An applicant

> shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams and formulas that fully set forth the claimed invention. Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was 'ready for patenting' such as by the disclosure of drawings of chemical formulas that show the invention was complete, or by describing distinguishing characteristics sufficient to show that he applicant was in possession of the claimed invention.

Applicants' written description clearly conveys that Applicants had possession of the instant invention at the time of filing. Applicants described the claimed invention with its limitations using words and formulas. Further, Applicants reduced the invention to practice. One of ordinary skill in the art of protein crystallography would understand the written description and claims as filed by Applicants. The application as originally filed provided adequate written description for the claims as originally filed. Applicants respectfully direct the Examiner's attention to M.P.E.P. §2163, which states:

> If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, **even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met**. See, e.g. *Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson*, 454 F.2d 746, 751, 172, USPQ 391, 395 (CCPA 1972) (emphasis added)

Applicants' written description, through text, formulas and working examples, convey that Applicants had possession of the invention at the time the instant application was filed.

With respect to 35 U.S.C. §112, first paragraph, Applicants have provided a written description of the invention at, *inter alia,* pages 5-43 of the instant specification. Applicants have shown how to make and use the invention on pages 43-47 wherein pharmaceutical formulations are described and in the Experimental section on pages 47-61 of the instant specification.

M.P.E.P. 2163 states:

> Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as the disclosure of drawing or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the

> claimed invention. See, e.g. *Pfaff v. Wells Elecs., Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406; *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed Cir 1991

Applicants have shown possession of the instant invention by reducing it to practice, as shown on pages 47, line 14 to page 61, line 10.

The Examiner alleges that the unbound Tie-2 polypeptide and the entire Tie-2 polypeptide and inhibitor III complex are encompassed in the "comprising" language used on line 3 of claim 21. Applicants respectfully point out that claim 21 is directed to a method of identifying inhibitors of Tie-2. The word "comprising" on line two of claim 21 refers to the steps of the method. With respect to the word "comprising" on line three of claim 21, as pointed out above in the response to the rejection of claims 21-27, 32 and 33 under 35 U.S.C. §112, first paragraph, step (a) of Claim 21 is limited to obtaining the atomic coordinates of a crystal of a polypeptide comprising the *catalytic domain* of a Tie-2 protein. Therefore, the use of at least those coordinates comprising the *catalytic domain* of a Tie-2 protein are the defining feature of the instant invention. The atomic coordinates of the catalytic domain is always present in the instant invention. It is not relevant whether other coordinates are present, so long as the atomic coordinates of the catalytic domain are included. Therefore, the word "comprising" is appropriate in that the polypeptide must contain catalytic domain of a Tie-2 protein but may include other sequences as well.

The term "comprising" is used because according to Landis on Mechanics of Patent Claim Drafting, Third Edition:

> Most ordinary combination claims require a transitional word or phrase between the preamble (naming the thing to be claimed) and the body of the claim (defining what the elements or parts of the thing are). Two recommended forms of transition that can be employed for most claims are the phrases: "which comprises" or "comprising". . . However, "comprising" is recommended simply because it has become a standardized word of the patent art.

Landis on Mechanics of Patent Claim Drafting, Third Edition, Robert C. Faber, March 1990, page 11.

The term "comprising" "is recognized as an open term that allows one to read on additional structures to the claim". *Special Metals Corporation v. Teledyne Industries, Inc.*, 219 USPQ 953

at 956 (4th Cir., 1983). However, the term "comprising" does not allow the incorporation into a claim, in which it is used, elements that would enlarge the scope of the claim, as stated by the Court of Appeals for the Federal Circuit (CAFC):

> The transitional phrase, which joins the preamble of a claim with the body of a claim, is a term of art and as such affects the legal scope of a claim. While a transitional term such as "comprising". . . does not exclude additional unrecited elements, or steps (in the case of a method claim), we conclude that the transitional phrase does not, in the present case, affect the scope of the particular structure recited within the method claim's step.

Moleculon Research Corp. v. CBS, Inc., 229 USPQ 805 at 812 (CAFC, 1986).

Therefore, the Examiner's allegation, that "comprising" leaves open the possibility of inclusion of other Tie-2 polypeptides, would mean that Applicants would be able to expand the scope of the claim. This is not a possibility which is contemplated by the mere use of the term "comprising" nor is the Examiner's allegation supported by the Specification. The basis for Applicants' claim 21 is found at page 2 of the Specification, nowhere does it mention the possibility of adding other Tie-2 polypeptides. The term "comprising" is merely a standardly recognized transitional term of patent drafting and does not make the claim in which it is used indefinite.

Based upon the foregoing, the rejection of Claims 21-27, 32 and 33 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time of the invention was filed, had possession of the claimed invention, is obviated and should be withdrawn.

The Examiner has rejected claims 21-27, 32 and 33 under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention. The Examiner alleges that claims 21-25 are vague and indefinite due to the unclarity of citing an abbreviation, such as Tie-2. Applicants respectfully traverse this rejection. Applicants submit as Exhibit A a copy of Shawver, Laura K. et al, DDT, Vol. 2, No. 2, February 1997, "Receptor tyrosine kinases as targets for inhibition of angiogenesis" as evidence that the term "Tie-2" is a well known term of art and was known prior to March 22, 2001, the filing date of the instant application.

The Examiner has rejected claim 32 alleging that there is insufficient antecedent basis for this limitation "the ligand" in the claim or in the claims from which it is dependent. Applicants

have amended claim 32 to overcome this rejection. Support for this amendment can be found, *inter alia*, at page 25, line 25 to page 26, line 1.

Based upon the foregoing, the rejection of claims 21-27, 32 and 33 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention is obviated and should be withdrawn.

The Examiner has rejected claims 21, 22 and 26 under 35 U.S.C. §103(a) as allegedly being unpatentable over Chen et al (P/N 6,160,092) in view of *In re Gulack* (703 F.2d 1381, 1385, 217 USPQ 401, 404 (Fed. Cir. 1983)). Applicants respectfully traverse this rejection.

The Examiner has not established a *prima facie* case of obviousness. In order to establish a *prima facie* case of obviousness, first there must be some suggestion or motivation to modify the reference. The reference does not provide any suggestion or motivation to modify Chen et al. to arrive at a method of identifying compounds that inhibit a Tie-2 protein. Second, there must be a reasonable expectation of success. One would not look to Chen et al., which describes work with STAT proteins, for guidance on crystallizing Tie-2 proteins.

The Court of Appeals for the Federal Circuit has stated the following on the issue of obviousness:

> Uniroyal, Inc. v. Rudkin-Wiley Corp., 837 F. 2d 1044, 1051-52, 5 USPQ 1434, 1438 (Fed. Cir. 1988), cert. denied, 109 S. Ct. 75 (1988), on remand, 13 USPQ2d 1192 (D. Conn. 1989) "Something in the prior art as a whole must suggest the desirability, and thus the obviousness, of making the combination."; In re Stencel, 828 F. 2d 751,755, 4 USPQ2d 1071, 1073 (Fed. Cir. 1987) obviousness cannot be established "by combining the teachings of the prior art to produce the claimed invention, absent some teaching or suggestion that the combination be made."' Alco Standard Corp. v. Tennessee Valley Authority, 808 F. 2d 1490, 1498, 1 USPQ2d 1337, 1343 (Fed. Cir. 1986), cert. dismissed, 108 S. Ct. 26 (1987) "the question is not simply whether the prior art 'teaches' the particular element of the invention, but whether it would 'suggest the desirability, and thus the obviousness, of making the combination.'"; Carella v. Starlight Archery, 804 F. 2d 135,231 USPQ 644 (Fed. Cir. 1986); ACS Hospital Sys., Inc. v. Montefiore Hospital, 732 F. 2d 1572, 221 USPQ 929 (Fed. Cir. 1984) "Obviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching or suggestion supporting the combination. Under section 103, teachings of references can be combined only if there is some suggestion or incentive to do so."

Donald S. Chisum, Patents, A Treatise on the Law of Patentability, Validity and Infringement, Vol. 2, 5-218, 1992.

In making a *prima facie* obviousness determination, an invention must be considered as a whole. In determining the differences between the prior art and the claims, the question under 35 U.S.C. §103 is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. Stratoflex, Inc. v. Aeroquip Corp., 713 F.2d 1530, 218 USPQ 871 (Fed. Cir. 1983); Schenck v. Nortron Corp.,713 F.2d 782, 218 USPQ 698 (Fed. Cir. 1983). The Examiner has not shown how Chen et al. renders obvious the method of Applicants' claim 21. Applicants' claim 21 includes the step of obtaining the atomic coordinates of a crystal of a polypeptide comprising the catalytic domain of a Tie-2 protein. Chen et al. is directed to the STAT protein. Further, there is no suggestion in Chen et al. how to arrive at the desired Tie-2 crystals. Applicants maintain that Chen et al. does not render claim 21 obvious.

To establish a *prima facie* case of obviousness, the invention must be considered as a whole, there must be some suggestion or motivation to modify the reference, the reference must teach or suggest all of the claim limitations and there must be a reasonable chance of success. The Examiner has not provided any motivation to modify Chen et al. Further, Chen et al. does not teach or suggest all of the limitations of Applicants' claims. As stated in M.P.E.P. 2143.03, "To establish prima facie obviousness of a claimed invention, all of the claim limitations must be taught or suggested by the prior art." In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). No such motivation or suggestion exists in Chen et al. When the prior art fails to suggest the claimed invention as a whole, as it does here, any reconstruction of the prior art to obtain that invention necessarily and inevitably requires impermissible hindsight.

Further, Claim 21 is a method claim directed to identifying a compound which is an inhibitor of a Tie-2 protein. Claim 21 recites specific steps which are to be used in this method. Chen et al. does not suggest, much less teach the steps claimed by the Applicants.

The Examiner alleges that "...an artisan of ordinary skill in the art would have been motivated to include any crystalline protein already identified into this method in order to search for new drugs. Therefore, it would have been obvious to one having ordinary skill in the art at the time the invention was made to include the three-dimensional model of the Tie-2 protein and Tie-2/Inhibitor III in the method, in order to search for possible drug candidates as described by Chen et al.." As discussed above, an invention is to be considered as a whole. The claimed invention may not be dissected into discrete elements to be analyzed in isolation, but must be

considered as a whole. See, e.g. W.L. Gore & Assoc. Inc. v. Garlock, Inc. 721 F.2d 1540, 1548, 220 USPQ 303, 309 (Fed. Cir. 1983)); Jones v. Hardy, 727 f.2d 1524, 1530, 220 USPQ 1021, 1026 (Fed. Cir. 1983).

The Examiner alleges that Chen et al. describes determining the three-dimensional structure of a compound based on structural coordinates obtained from X-ray crystallographic analysis of crystals, various binding domains of a protein, interactive areas in these domains using crystal structure data and catalytic sites, using computer modeling to select potential agents and contacting the agents with the protein, determining whether the agent affects the ability of the protein to induce expression of a gene that is operably under the control of a promoter containing the binding site for the protein, that potential modulators can be synthesized de novo or selected from a library of chemicals, that proteins and core fragments thereof may be chemically synthesized and modified, identifying potential modulators by screening a random peptide library and further modified using computer modeling programs. Chen et al. is directed to a method of identifying a compound which is an inhibitor of a STAT protein, whereas the instant application is directed to a method of identifying a compound which is an inhibitor of a Tie-2.

The Examiner alleges that "Even though the method described by Chen et al. does not specify that the active site was identified by the crystal structure coordinates and the three-dimensional model of the Tie-2 protein and Tie-2/Inhibitor III complex, the specific limitations of crystal structure coordinates and the three-dimensional model of the Tie-2 protein and Tie-2/Inhibitor III complex in this instant do not distinguish the invention from the prior art in terms of patentability, because they are nonfunctional descriptive subject matter". The Examiner cites *In re Gulack* to support this position, alleging that the coordinate data set derived from the crystal structure of the Tie-2 protein or Tie-2/Inhibitor III complex to develop three-dimensional models in the instant case are merely stored so as to be read or outputted by a computer without creating any functional interrelationship...". Applicants respectfully disagree. In *In re Gulack*, the court found that the printed digits of Gulack's invention were functionally related to the band. In the instant invention, as in *Miller*, 57 CCPA 809, 418.F2d 1392, 14 U.S.P.W. (BNA) 46, there is a functional relationship between the printed matter and the invention. In the instant invention, without the atomic coordinates to Tie-2 one cannot define the active subsites or identify a compound which will bind to said subsites of Tie-2. The atomic coordinates are not

nonfunctional descriptive subject matter but are an essential component in Applicants' method of identifying an inhibitor of Tie-2. The atomic coordinates are specific to each protein. That is, for each protein the atomic coordinates are different. Atomic coordinates for STAT are different from those for Tie-2. In order to obtain the atomic coordinates for a particular protein one must start with that protein.

Based upon the foregoing, the rejection of claims 21, 22 and 26 under 35 U.S.C. §103(a) over Chen et al. in view of *In re Gulack* is obviated and should be withdrawn.

The Examiner has rejected claims 21-27 under 35 U.S.C. §103(a) as allegedly being unpatentable over Chen et al (P/N 6,160,092) in view of *In re Gulack* (703 F.2d 1381, 1385, 217 USPQ 401, 404 (Fed. Cir. 1983)), *In re Best* (195 USPQ 430) and *In re Fitzgerald* (205 USPQ 594) and Ziegler (P/N 5,447,860). Applicants respectfully traverse this rejection.

The Examiner has failed to present a *prima facie* case of obviousness. In order to establish a *prima facie* case of obviousness, first there must be some suggestion or motivation to modify the reference. Neither Chen et al. nor Ziegler provide any suggestion or motivation to modify Chen et al. to arrive at a method of identifying compounds that inhibit a Tie-2 protein by obtaining the atomic coordinates of a crystal of a polypeptide comprising the catalytic domain of a Tie-2 protein. Second, there must be a reasonable expectation of success. One would not look to Chen et al., which describes work with STAT proteins, for guidance on crystallizing Tie-2 proteins. With respect to Ziegler, one would not look to Ziegler, which describes the biological ligand of Tie that binds to the extracellular domain, to make small molecule ligands that bind to the *catalytic domain* of Tie-2.

In making a *prima facie* obviousness determination, an invention must be considered as a whole. The Examiner has not presented arguments as to how Chen et al. and Ziegler make obvious Applicants' method of identifying a compound which is an inhibitor of a Tie-2 protein using a polypeptide comprising the catalytic domain of a Tie-2 protein. As stated above in the argument regarding the rejection of claims 21, 22 and 26 under 35 U.S.C. §103(a) as allegedly being unpatentable over Chen et al. in view of *In re Gulack* (supra), in determining the differences between the prior art and the claims, the question under 35 U.S.C. §103 is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. As stated in M.P.E.P. 2143.03, "To establish prima facie obviousness of a claimed invention, all of the claim limitations must be taught or suggested by

the prior art." In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). Neither Chen et al. nor Ziegler teach the limitation of the catalytic domain. When the prior art fails to suggest the claimed invention as a whole, as it does here, any reconstruction of the prior art to obtain that invention necessarily and inevitably requires impermissible hindsight.

The Examiner asserts that Chen et al. describes determining the three-dimensional structure of a compound based on structural coordinates obtained from X-ray crystallographic analysis of crystals, various binding domains of a protein, interactive areas in these domains using crystal structure data and catalytic sites, using computer modeling to select potential agents and contacting the agents with the protein, determining whether the agent affects the ability of the protein to induce expression of a gene that is operably under the control of a promoter containing the binding site for the protein, the potential modulator can be synthesized de novo or selected from a library of chemicals, proteins and core fragments thereof may be chemically synthesized, identifying potential modulators by screening a random peptide library and further modified using computer modeling programs. As stated above in response to the rejection of claims 21, 22 and 26 under 35 U.S.C. §103(a) as allegedly being unpatentable over Chen et al. in view of *In re Gulack* (supra), Chen et al. is directed to a method of identifying a compound which is an inhibitor of a STAT protein, whereas the instant application is directed to a method of identifying a compound which is an inhibitor of a Tie-2.

The Examiner acknowledges that Chen et al. does not describe the three-dimensional structure of the Tie-2 or Tie-2/Inhibitor III complex, various characteristics of the Tie-2 protein, or the amino acid sequence of SEQ ID No: 1. The Examiner alleges that "Even though the method described by Chen et al. does not specify that the active site was identified by the crystal structure coordinates and the three-dimensional model of the Tie-2 protein and Tie-2/Inhibitor III complex, the specific limitations of crystal structure coordinates and the three-dimensional model of the Tie-2 protein and Tie-2/Inhibitor III complex in this instant do not distinguish the invention from the prior art in terms of patentability, because they are nonfunctional descriptive subject matter". The Examiner cites *In re Gulack* to support this position, alleging that the coordinate data set derived from the crystal structure of the Tie-2 protein or Tie-2/Inhibitor III complex to develop three-dimensional models in the instant case are merely stored so as to be read or outputted by a computer without creating any functional interrelationship...". Applicants respectfully disagree. In *In re Gulack*, the court found that the printed digits of Gulack's

invention were functionally related to the band. In the instant invention, as in *Miller*, 57 CCPA 809, 418.F2d 1392, 14 U.S.P.W. (BNA) 46, there is a functional relationship between the atomic coordinates and the invention. In the instant invention, without the atomic coordinates to Tie-2 one cannot define the active subsites or identify a compound which will bind to said subsites of Tie-2. The atomic coordinates are not nonfunctional descriptive subject matter but are an essential component in Applicants' method of identifying an inhibitor of Tie-2. The atomic coordinates are specific to each protein, that is, for each protein they are different. Atomic coordinates for STAT are different from those for Tie-2. In order to obtain the atomic coordinates for a particular protein one must have the proper protein.

The Examiner alleges that Ziegler describes a polypeptide sequence of a receptor tyrosine kinase (Fig. 1f-1h, residues 802-1124) that is identical to residues 802-1124 of SEQ ID NO:1 of the instant invention as stated in claim 27. The Examiner further alleges that Ziegler describes that ork can be used as a research tool for identifying ligands and assessing the biological effects of ligand binding (col. 17, lines 26-55) as state in claim 22. With respect to *In re Best* and *In re Fitzgerald*, the Examiner asserts that these cases discuss the support of rejections wherein the prior art discloses subject matter which there is reason to believe inherently includes functions that are newly cited or is identical to a product instantly claimed. In such a situation the burden is shifted to the applicants to "prove that the subject mater shown to be in the prior art does not possess characteristic relied on". Applicants respectfully disagree that Ziegler discloses subject matter that inherently includes functions that are newly cited or is identical to Applicants' invention. The context in which Zieger discloses that ork can be used as a research tool for identifying ligands and assessing the biological effects of ligand binding make it clear that Ziegler refers to the biological ligand of Tie that binds to the extracellular domain, not *small molecule* ligands that bind to the *catalytic domain* of Tie-2 as Applicants' invention does. Applicant's invention is directed to a method of identifying a compound which is an inhibitor of a Tie-2 protein. This method comprises obtaining the atomic coordinates of a crystal polypeptide comprising the *catalytic domain* of a Tie-2 protein. Protein kinase catalytic domains have been defined with fairly precise boundaries, as described in Hanks et al, Science (241):42-52 (1998), a copy of which is attached as Exhibit B for the Examiner's convenience. Ziegler has not disclosed either subject matter that is inherent in or identical to Applicants' invention.

Applicants, in compliance with *In re Best* and *In re Fitzgerald*, have distinguished the instant invention from that disclosed in Ziegler.

Claim 21 is directed to a method of identifying a compound and it includes three specific steps: obtaining atomic coordinates of a crystal of a polypeptide comprising the catalytic domain of a Tie-2 protein, using those atomic coordinates to define the active subsites of Tie-2 and identifiying a compound which binds to one or more active subsites and is an inhibitor of a Tie-2 protein. Chen et al. does not teach or suggest the first step of Applicants' method because Chen et al. is directed to work with crystals comprising a core portion of a STAT, which includes two *SH2 domains*, and a duplex DNA, not a crystal of a polypeptide comprising the *catalytic domain* of a Tie-2 protein. Chen et al. also does not teach or suggest the second step of Applicants' method because Chen et al. does not teach or suggest using the atomic coordinates to define the active subsites of Tie-2. Chen et al. does not teach or suggest the third step of Applicants' method because Chen et al. does not teach how to identify compounds that bind to the active subsites or are inhibitors of a Tie-2 protein. Instead, Chen et al. is directed to identifying compounds that enhance or diminish the ability of a STAT to induce the expression of a gene operably under the control of a promoter containing a binding site for the STAT.

Ziegler does not teach or suggest the first or second step of Applicants' method because Ziegler does not teach obtaining atomic coordinates or the use thereof. Ziegler also does not teach the third step of Applicants' method because Ziegler does not teach identifying compounds that bind to the active subsites of Tie-2.

Based upon the foregoing, the rejection claims 21-27 under 35 U.S.C. §103(a) as allegedly being unpatentable over Chen et al (P/N 6,160,092) in view of *In re Gulack* (703 F.2d 1381, 1385, 217 USPQ 401, 404 (Fed. Cir. 1983)), *In re Best* (195 USPW 430) and *In re Fitzgerald* (205 USPQ 594) and Ziegler (P/N 5,447,860) is obviated and should be withdrawn.

No fees are due for the instant amendment since the total number of claims after entry of the amendments hereinabove is not more than the total number of claims that Applicants have paid for to date.

Based upon the foregoing, Applicants believe that claims 21-27, 32 and 33 are in condition for allowance. Prompt and favorable action is earnestly solicited.

If the Examiner believes that a telephone conference would advance the condition of the instant application for allowance, Applicants invite the Examiner to call Applicants' agent at the number noted below.

Respectfully submitted,

Date: December 23, 2003

Gayle O'Brien

Gayle B. O'Brien
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Exhibit A

Drug Discovery Today

Receptor tyrosine kinases as targets for inhibition of angiogenesis

Laura K. Shawver, Kenneth E. Lipson, T. Annie T. Fong, Gerald McMahon, Greg D. Plowman and Laurie M. Strawn

Anti-angiogenic agents potentially have broad applications in the clinic. Although most agents now in development are intended ultimately for use as anti-cancer drugs, patients with a range of disorders may benefit in the longer term. The signal recognition and transduction processes involved in controlling angiogenesis are complex and are likely to be dependent on the status of the target endothelial cell in a specific organ or tissue. In this review, the authors focus on signaling interactions that affect microvascular endothelium and the role of growth factors and their receptor tyrosine kinases in the regulation of microvessel physiology as they relate to the angiogenic process.

Angiogenesis, the sprouting of capillaries from pre-existing blood vessels, is a complex process involving many biological and cellular functions. The process begins when endothelial cells become activated and cause dissolution of the basement membrane, leading to migration of the endothelial cells. New capillary lumina are formed by realignment and vacuolization of the migrating endothelial cells. Capillary loops are then formed, followed by the deposition of new basement membranes around the vessels (for review, see Ref. 1). These events are illustrated in Figure 1. All of these processes depend on the tight regulation of factors that 'promote' or 'inhibit' these biological events. Signal recognition and transduction are complex processes that are likely to be dependent on the status of the target endothelial cell in a specific organ or tissue. This review will focus on signaling interactions that affect microvascular endothelium and will not address large vessel endothelium, which is important for controlling vasoconstriction, vasodilation, blood pressure and other physiological parameters that affect blood supply. In addition, the review will primarily address the role of growth factors and their receptor tyrosine kinases (RTKs) in the regulation of microvessel physiology as they relate to the angiogenic process.

During development, the angiogenic process is active to ensure the formation of the capillary network associated with developing organ systems. However, the turnover of endothelial cells in the normal human adult is very low, in the order of years, except during formation of the corpus luteum, pregnancy, wound healing following tissue injury, or when oxygen supply is compromised. Pathologic angiogenesis occurs under many conditions and is thought to be induced by local ischemia. Diseases in which angiogenesis is thought to play a critical role in the underlying pathology include: ocular diseases such as diabetic retinopathy, retinopathy of prematurity and age-related macular degeneration; vascular diseases such as ischemic heart disease and atherosclerosis; chronic inflammatory disorders such as psoriasis and rheumatoid arthritis; and solid tumor growth.

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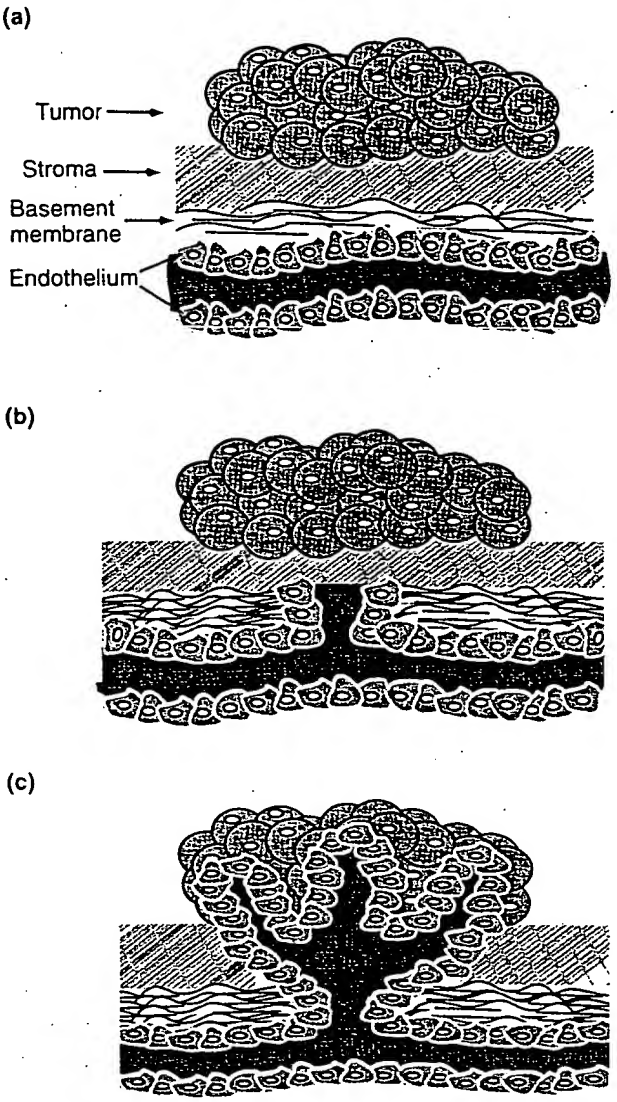

Figure 1. Formation of new blood vessels by angiogenesis. Angiogenesis, the sprouting of new blood vessels from pre-existing ones, is a complex mechanism involving several biological processes as outlined in the text. (a) Small nonangiogenic tumor; (b) invasion and migration of endothelial cells through the basement membrane; (c) extension of vessels into the tumor.

While RTKs are thought to be important in angiogenesis associated with these pathologic conditions, this review will primarily focus on the role of RTKs in tumor angiogenesis.

Angiog nesis in solid tumor growth

Results of research originating in the 1970s and continuing today have led to the conclusion that new blood vessel growth is required for the growth and metastasis of solid tumors. Immunohistochemical analysis of sections from growing tumors shows a preponderance of blood vessels, irrespective of tumor type. This is illustrated in Figure 2. These new blood vessels are required for tumors to expand beyond a minimum volume. Before tumors acquire the angiogenic phenotype, new blood vessel growth is kept in check by a balance of angiogenic and anti-angiogenic factors. Some of these factors are listed in Box 1.

It has been proposed recently by Hanahan and Folkman[2] that there is a 'switch' that perturbs the balance between these factors. The 'angiogenic switch' has been suggested to be a component of the tumor phenotype that is often activated during the preneoplastic stage in tumor development. With the balance disturbed, unchecked angiogenic factors released from hypoxic tumor cells migrate to nearby blood vessel endothelia, which signals the activation of biochemical events leading to the cellular changes associated with the angiogenic process. This is illustrated in Figure 3.

While tumors that lack adequate vasculature become necrotic[3] and/or apoptotic[4,5], tumors that have undergone neovascularization may not only enter a phase of rapid growth but may also have increased metastatic potential. The significance of angiogenesis in human tumors has been highlighted by recent studies that relate the angiogenic phenotype to patient survival. These studies found that the number of microvessels in a primary tumor has prognostic significance in breast carcinoma[6,7], bladder carcinomas[8], colon carcinomas[9] and tumors of the oral cavity[10].

Receptor tyrosine kinases (RTKs)

RTKs (also known as growth factor receptors) play an important role in many cellular processes. They make up a large class of receptors represented by at least 19 distinct subfamilies (for review, see Ref. 11). All of these molecules have an extracellular ligand-binding domain, a transmembrane domain and a tyrosine kinase domain. Upon ligand binding, receptors dimerize, the tyrosine kinase is activated and the receptors become autophosphorylated (for review, see Ref. 12). Receptor-specific phosphotyrosines serve as binding sites for other substrates. Some of these molecules are in turn phosphorylated, resulting in their activation and the ability to affect additional downstream molecules. Others do not have intrinsic enzymatic activity but serve as docking proteins for enzymes that are activated only when brought into association with the appropriate cellular compartment. The cascade triggered by RTK activation

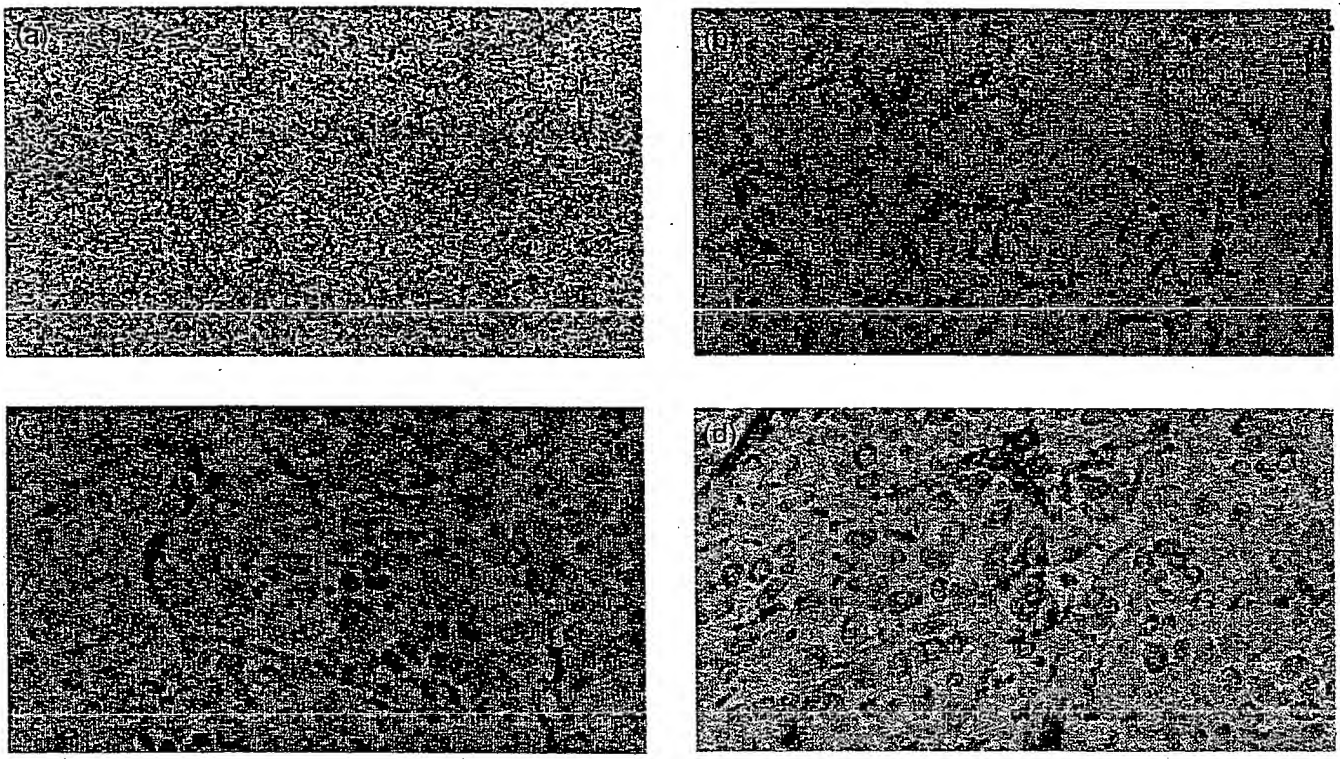

Figure 2. Immunohistochemical detection of blood vessels in solid tumors. Endothelial cells were stained with an anti-PECAM-1 antibody. (a,b) EpH4-VEGF mouse epithelial cells engineered to express VEGF; (c) Calu-6 human lung carcinoma; (d) A-431 human epidermoid carcinoma; (e) RF-48 human metastatic gastric adenocarcinoma. Magnifications: ×100 (a); ×200 (b–e).

modulates cellular events, determining proliferation, differentiation and morphogenesis in a positive or negative fashion.

Disturbances in the expression of growth factors, their cognate RTKs, or constituents of downstream signaling pathways are commonly associated with many types of cancer. Gene mutations giving rise to altered protein products have been shown to alter the regulatory mechanisms influencing cellular proliferation, resulting in tumor initiation and progression.

Uncontrolled growth responses are manifested via both autocrine and paracrine pathways. As shown in Figure 3, the paracrine pathway is thought to be predominant for angiogenesis. Growth factors released by tumor cells begin the signaling cascade that regulates gene transcription (Figure 3a) for those proteins involved in new blood vessel formation (Figure 3b).

RTKs in angiogenesis

There are several strategies for demonstrating the role of RTKs in angiogenesis. Early work usually focuses on defining a temporal and spatial correlation of ligand and receptor expression with biological events in model systems for angiogenesis, including wound healing, tumor growth and induced corneal angiogenesis. The role of RTKs in the angiogenic process can be better understood, however, by

examining the cellular phenotype following interference with receptor signaling. This can be achieved through several techniques, described below.

One strategy for interfering with receptor signaling is to inhibit ligand binding. This can be accomplished with specific receptor-binding antagonists such as ligand fragments, or with nonspecific antagonists such as suramin, with neutralizing antibodies to either the ligand or receptor, or with an excess of soluble receptor or ligand-binding protein, which will sequester the ligand.

A second strategy for interfering with receptor signaling is to block signal transduction by overexpression of a dominant-negative receptor. Because receptor kinases typically dimerize to induce signal transduction through transphosphorylation (for review, see Ref. 13), prevention of receptor dimerization due to overexpression of kinase-deficient receptors will attenuate activation of signaling. Receptors can be made kinase-deficient by introduction of a point mutation in amino acids critical for kinase function, or deletion of the kinase or entire cytoplasmic domain. Dominant-negative receptors attenuate signaling by forming heterodimers with endogenous receptors, which may allow partial signaling, and by diluting the number of effective growth factor binding sites with inactive homodimers of the dominant-negative receptor.

The third strategy for understanding receptor function involves depleting the receptor protein. This can be accomplished by the introduction of exogenous agents such as antisense oligonucleotides, antisense RNA, or ribozymes, all of which lead to degradation of the receptor mRNA and gradual depletion of the protein in the cell. Alternatively, embryos, and perhaps animals, can be made that lack the receptor or ligand of interest by homologous recombination in embryonic stem cells and inactivation of the target locus in offspring following implantation. Because mice with such targeted gene deletions ('knockouts') are often not viable, it is most common to compare embryos at various stages of development with normal embryos for altered development of vascular systems.

Box 1. Angiogenic and anti-angiogenic factors involved in the regulation of new blood vessel growth in tumors

Angiogenic factors	Anti-angiogenic factors
Basic fibroblast growth factor (bFGF)	Platelet factor 4
Acidic fibroblast growth factor (aFGF)	Thrombospondin-1
Transforming growth factor-a (TGF-a)	Transforming growth factor-b
Transforming growth factor-b (TGF-b)	Interferon-a
Platelet-derived growth factor (PDGF)	Prolactin fragment
Insulin-like growth factor (IGF)	Angiostatin (fragment of plasminogen)
Vascular endothelial growth factor (VEGF)	Tissue inhibitors of metalloproteinases
Platelet-derived endothelial cell growth factor	TIMP-1
Granulocyte colony-stimulating factor	TIMP-2
Hepatocyte growth factor (HGF)	TIMP-3
B61	bFGF soluble receptor
Tie-2 ligand	Placental proliferin-related protein
Angiogenin	
Tumor necrosis factor-a (TNF-a)	
Growth hormone	
Placental growth factor	
Interleukin 8	
Proliferin	
Prostaglandins E_1 and E_2	

Using these strategies, a number of RTKs have been shown to be involved in angiogenesis, either directly or indirectly (Figure 4). Of particular interest are Flt-1 and Flk-1, the receptors for vascular endothelial growth factor (VEGF), as well as Tie-1 and Tie-2. Flt-1 and Flk-1 are also known as VEGFR1 and VEGFR2, respectively; the human homolog of Flk-1 is KDR. For the purposes of this review, the VEGF receptors will be referred to as Flt-1 and Flk-1. These receptors, as well as Tie-1 and Tie-2, which are also referred to as TIE (tyrosine kinase with immunoglobin and EGF homology domains) and TEK (tunica interna endothelial cell kinase), are expressed primarily on endothelial cells and play a direct role in angiogenesis. A summary of these endothelial-specific RTKs and their ligands is shown in Figure 5.

Other RTKs of potential interest in angiogenesis include the epidermal growth factor (EGF) receptor, the platelet-derived growth factor (PDGF) receptor, the fibroblast growth factor (FGF) receptor family, c-met proto-oncogene product (MET) and epithelial cell kinase (ECK). They have also been implicated in angiogenesis, but they have broader expression patterns that encompass other cell types as well as endothelial cells. The following is an overview of the

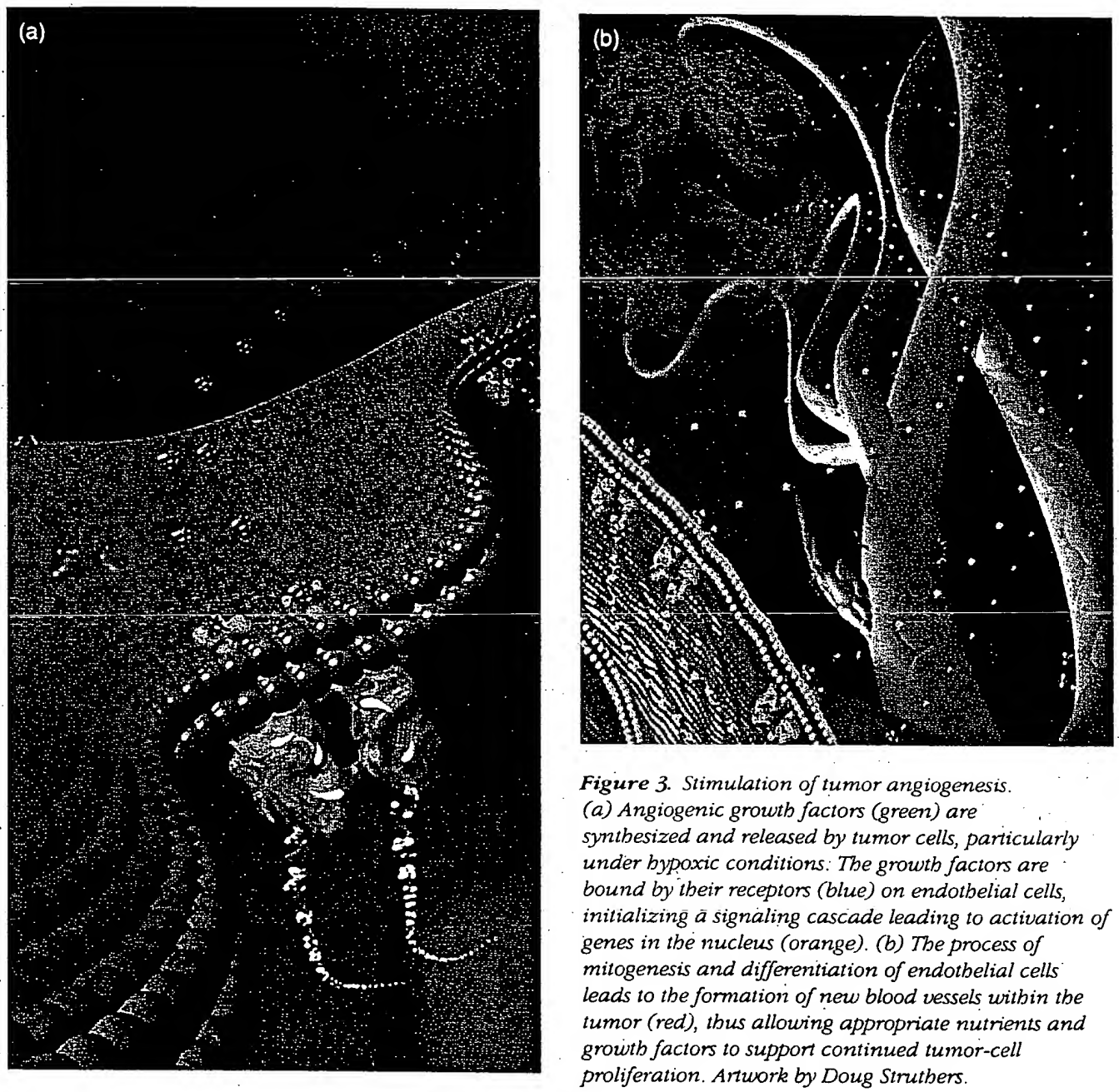

Figure 3. *Stimulation of tumor angiogenesis. (a) Angiogenic growth factors (green) are synthesized and released by tumor cells, particularly under hypoxic conditions. The growth factors are bound by their receptors (blue) on endothelial cells, initializing a signaling cascade leading to activation of genes in the nucleus (orange). (b) The process of mitogenesis and differentiation of endothelial cells leads to the formation of new blood vessels within the tumor (red), thus allowing appropriate nutrients and growth factors to support continued tumor-cell proliferation. Artwork by Doug Struthers.*

RTKs postulated to play a role in angiogenesis and a summary of the evident data.

VEGF receptors

mRNA for the receptor tyrosine kinases Flk-1 and Flt-1, and their ligand, VEGF (also known as VPF), is expressed in endothelial cell precursors and later in the endothelial cells of vessels throughout mouse and rat embryos[14–17]. Furthermore, protein expression of VEGF receptors was confirmed along the lumina of vessels in rat embryos by binding of ^{125}I-labeled VEGF (Ref. 18). Thus, the temporal and spatial patterns of the expression of VEGF and its receptors support their involvement in angiogenesis during development.

VEGF, Flt-1 and Flk-1 have also been implicated in the angiogenesis that occurs in many solid tumors, including gliomas[19,20], breast cancer[21], bladder cancer[22], colon carcinoma[9,23] and other cancers of the gastrointestinal tract[24]. A correlation has been observed between VEGF expression and vessel density in human breast tumors[7,25],

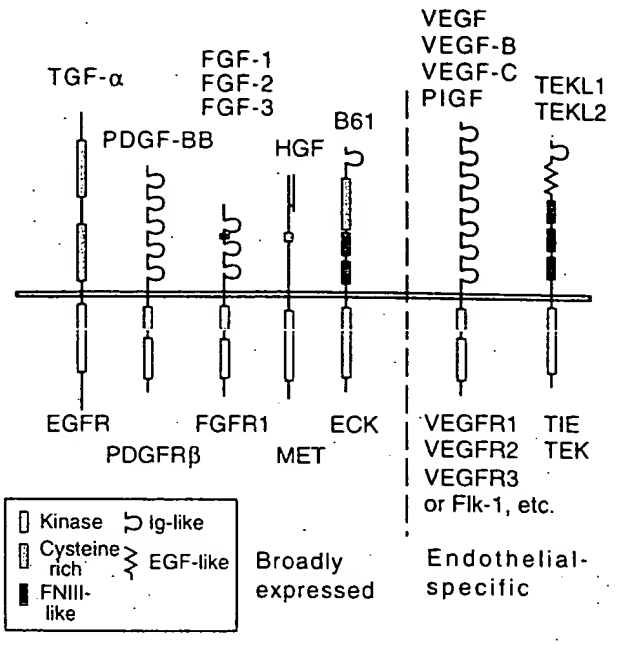

Figure 4. *Receptor tyrosine kinases (RTKs) in angiogenesis. Multiple sequence alignments of tyrosine kinase catalytic domains allow RTKs to be classified into distinct families. RTKs implicated in angiogenesis are shown. The horizontal line represents the plasma membrane with the extracellular domain above and the cytoplasmic domain below. TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor. PDGF-BB, platelet-derived growth factor; PDGFRβ, PDGF β-receptor. FGF-1, -2, -3, fibroblast growth factor; FGFR1, FGF receptor type 1. HGF, hepatocyte growth factor; MET, HGF receptor. B61, ligand for ECK; ECK, epithelial cell kinase. VEGF, -B, -C, vascular endothelial growth factor; PlGF, placental growth factor; VEGFR1, -2, -3 (Flk-1, etc.), VEGF receptors. TEKL1, -2, Tie-2 ligands; TIE, tyrosine kinase with immunoglobin and EGF homology domains; TEK, tunica interna endothelial cell kinase. FNIII, fibronectin type III repeat.*

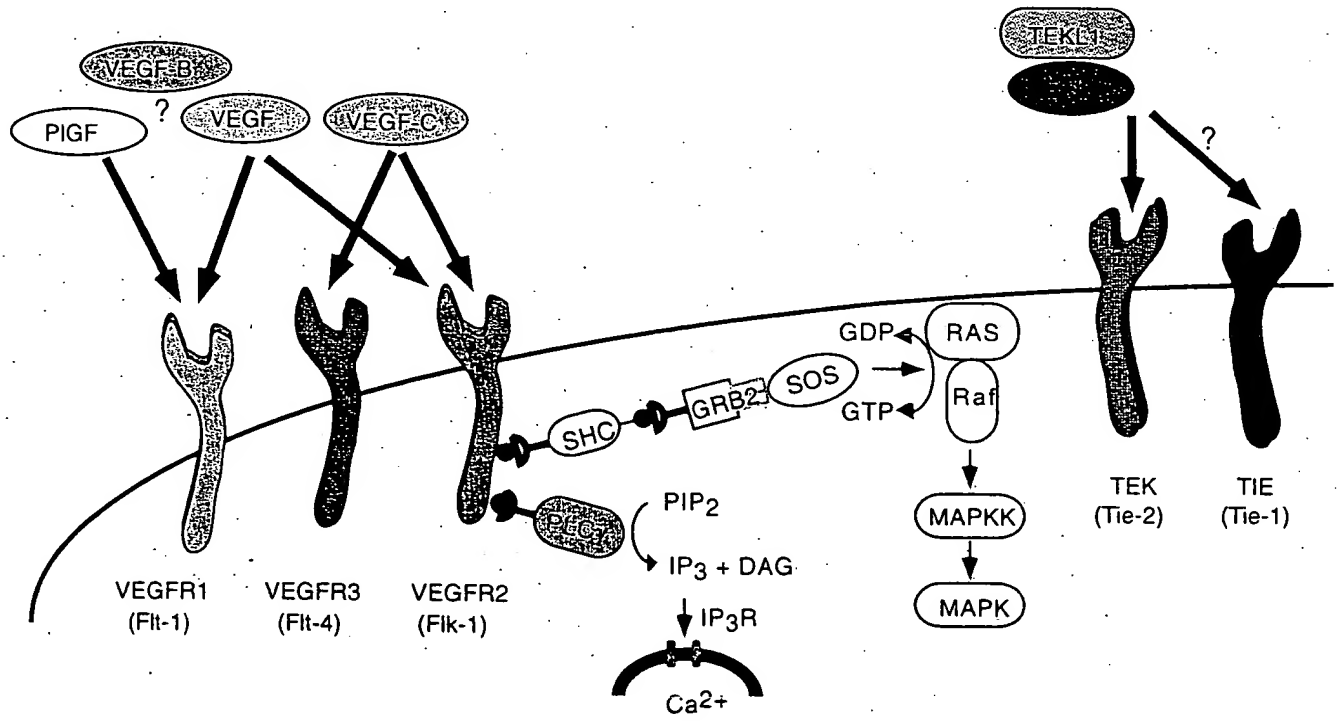

Figure 5. *Signaling through Flk-1 and Tie-2 family receptors. The three known vascular endothelial growth factor receptors (VEGFR1, -2, -3) have differential binding affinities for the various members of the VEGF family. Two Tie-2 ligands have recently been discovered (TEKL1, TEKL2). Ligand binding leads to receptor dimerization and activation, and the resulting phosphotyrosines on the receptors serve as binding sites for other proteins. Typical signal transduction pathways are illustrated.*

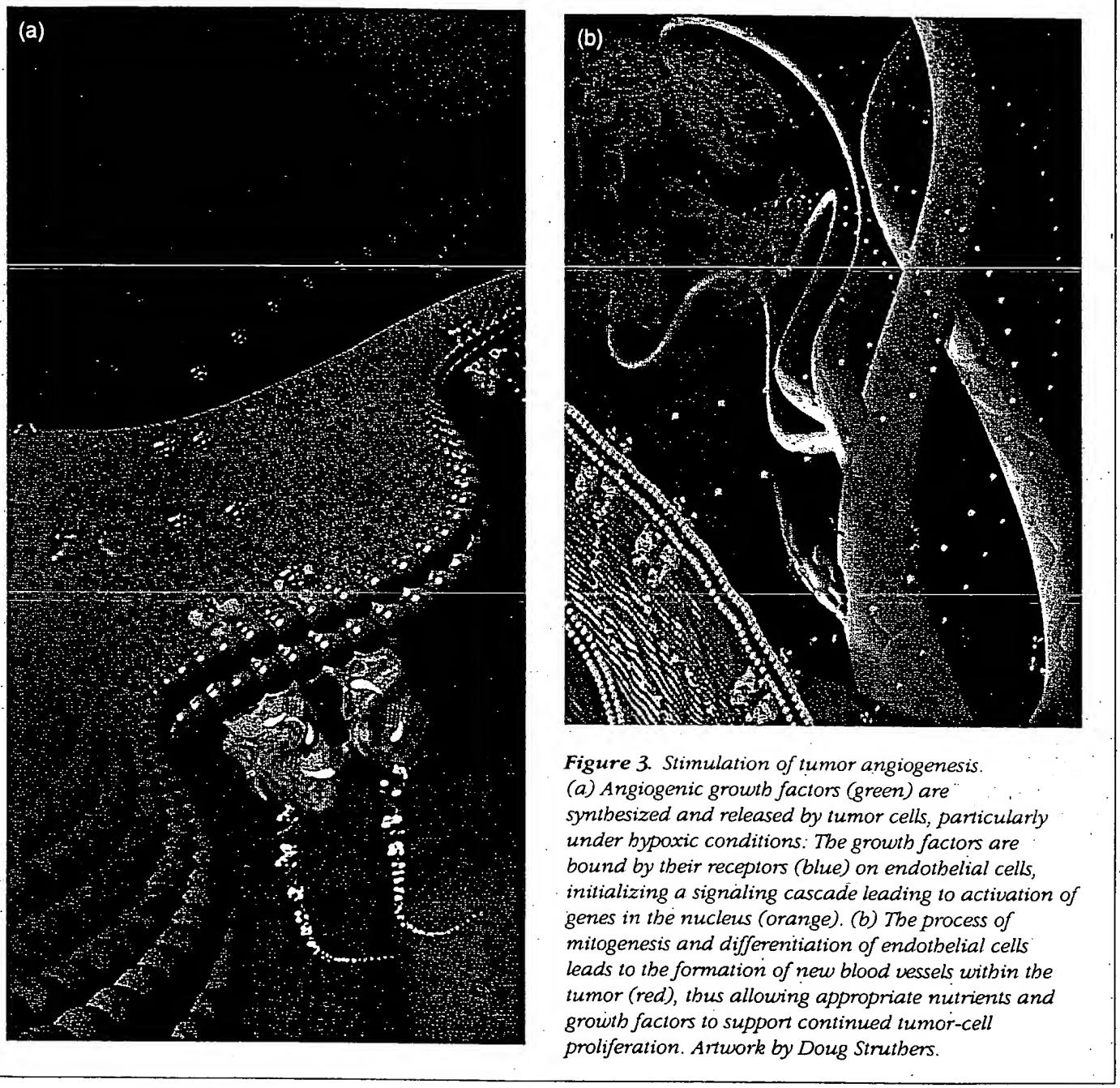

Figure 3. Stimulation of tumor angiogenesis. (a) Angiogenic growth factors (green) are synthesized and released by tumor cells, particularly under hypoxic conditions. The growth factors are bound by their receptors (blue) on endothelial cells, initializing a signaling cascade leading to activation of genes in the nucleus (orange). (b) The process of mitogenesis and differentiation of endothelial cells leads to the formation of new blood vessels within the tumor (red), thus allowing appropriate nutrients and growth factors to support continued tumor-cell proliferation. Artwork by Doug Struthers.

RTKs postulated to play a role in angiogenesis and a summary of the evident data.

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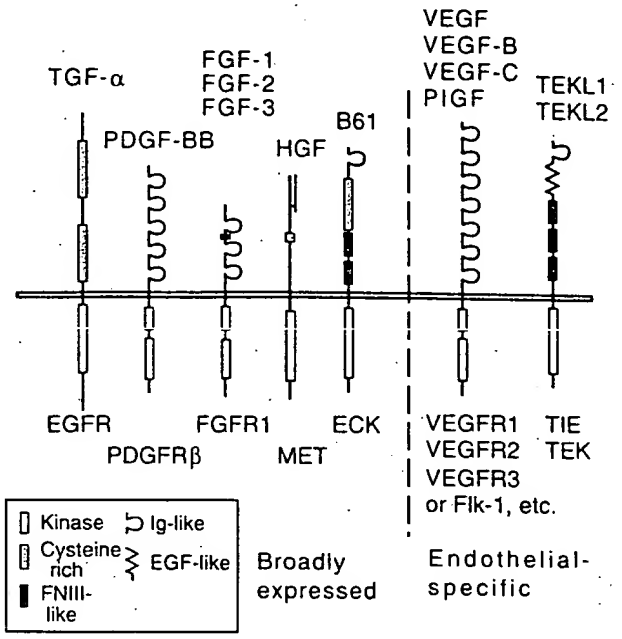

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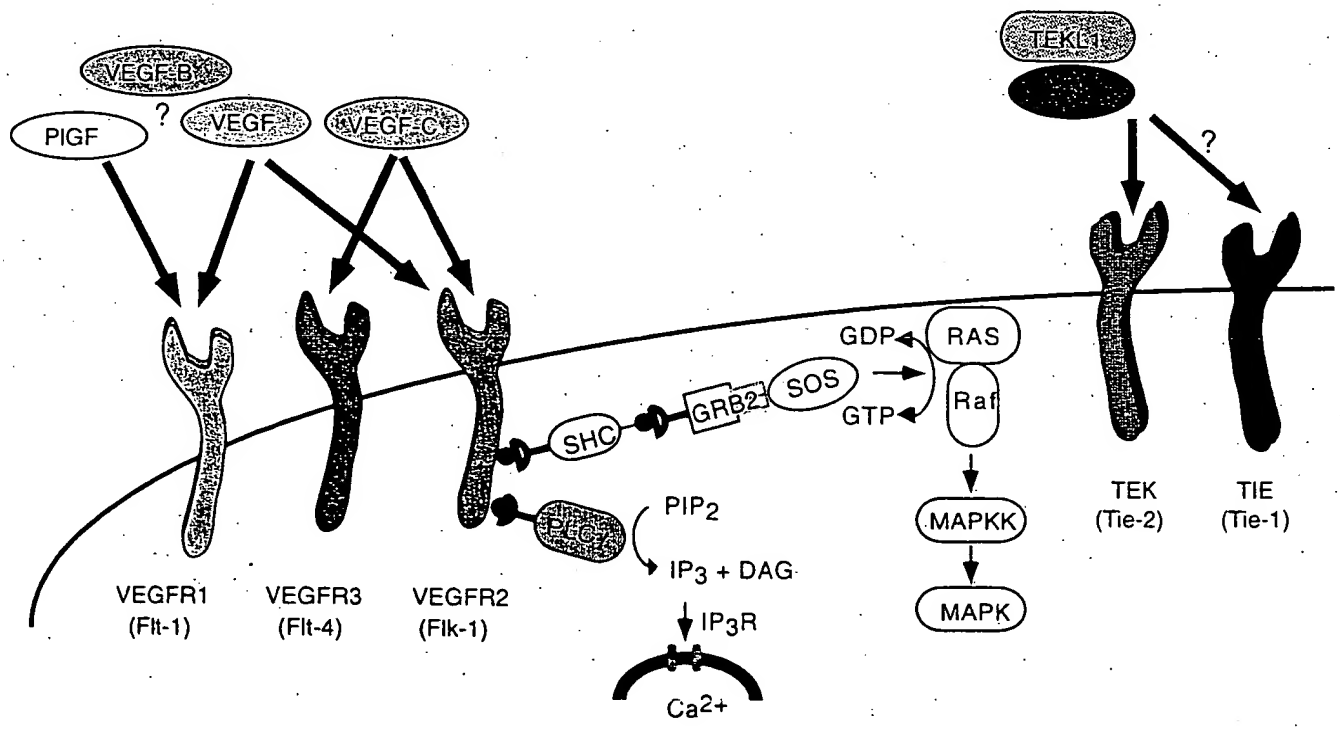

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renal cell carcinoma[26] and colon cancer[9]. In highly vascularized glioblastoma, transcripts for all three proteins were identified by *in situ* hybridization. Flk-1 was found to be dramatically upregulated, localized to the necrotic areas, when compared with low-grade gliomas. The receptors were detected in the endothelial cells of the vessels, whereas the VEGF was found in the tumor cells. None of the mRNAs were expressed in normal brain tissue in which angiogenesis does not occur[19,20].

VEGF is mitogenic for endothelial cells *in vitro*. In such a system, a neutralizing antibody against Flk-1 inhibited mitogenesis[27], as did a truncated soluble form of Flt-1, which competed for binding of VEGF to its receptors[28]. Similarly, ribozymes that cleave *flk-1* or *flt-1* mRNAs reduced the growth of human microvasculature endothelial cells, presumably by decreasing the amount of receptors on the cells[29]. However, mutant forms of VEGF that preferentially bind to Flt-1 do not stimulate endothelial cell proliferation, which raises questions regarding the role of Flt-1 in angiogenesis[30].

The genes for VEGF and its receptors have been disrupted through targeted mutagenesis in mice. Embryos homozygous for mutant *flk-1*, *flt-1* or VEGF were resorbed by days 10–12 of development. In the case of *flk-1* disruption, no endothelial cells or vessels were observed in the embryo or yolk[31]. This indicates that Flk-1 is required for development of mature endothelial cells. In contrast, embryos lacking Flt-1 had mature endothelial cells, but the vessels were large and disorganized[32]. In two studies with disruption of the VEGF gene, heterozygous as well as homozygous embryos were resorbed[33,34]. Mature endothelial cells were detected before resorption, but the vessels in the embryos and yolks were abnormal. Possibly, the newly identified members of the VEGF family, VEGF-B (Ref. 35) or VEGF-C/VRP (Refs 36,37) (Figure 5), can substitute for VEGF and allow maturation of endothelial cells, but cannot substitute for VEGF in later steps of angiogenesis.

A variety of techniques have been used to investigate the role of VEGF signaling in tumor angiogenesis. Dominant-negative Flk-1 lacking the kinase domain blocked the activation of the endogenous Flk-1 tyrosine kinase in cultured cells[38]. It also inhibited the growth of eight out of nine types of tumors implanted subcutaneously into nude mice, and significantly reduced vessel density in the small tumors that did form[38,39]. Furthermore, embryonic stem cells with disrupted VEGF grew very poorly as subcutaneous implants in nude mice compared with control embryonic stem cells[34]. Also, a reduction in VEGF expression following the introduction of antisense DNA constructs inhibited the growth of C6 rat glioma cells in nude mice as well as reducing vessel density within the tumor[40]. Human melanoma cells in nude/SCID mice were also inhibited using antisense constructs[41]. Likewise, reduction of VEGF levels with neutralizing antibodies inhibited the growth of human rhabdomyosarcoma, glioblastoma multiforme and leiomyosarcoma in Beige nude/xid mice[42], and of fibrosarcoma in BALB/c nude mice[43]. Thus, there is strong evidence that VEGF signaling through the Flk-1 tyrosine kinase is required for angiogenesis in solid tumor growth as well as in development.

Tie-1 and Tie-2

Tie-1 and Tie-2 (TEK) are receptor kinases whose expression is most prevalent in the vascular endothelium during embryonic development[44–46]. In adults, the Tie receptors are weakly expressed, but are induced during active angiogenesis. For example, Tie expression is upregulated in skin capillaries during wound healing[47] and in angiogenesis associated with metastatic melanomas[48]. Transgenic mouse embryos expressing a dominant-negative Tie-2 receptor were developmentally delayed, had compromised heart development and exhibited signs of hemorrhage[49], suggesting that Tie-2 kinase activity is important for vasculogenesis. Mouse embryos lacking Tie-1 (Refs 50, 51) or Tie-2 (Refs 49, 51) exhibited somewhat different phenotypes. Embryos of Tie-1 knockout mice died in mid- to late gestation or shortly after birth as a result of breathing problems. All embryos of mice lacking Tie-1 exhibited peripheral and abdominal hemorrhage and/or edema. Because their vasculature appeared to be properly developed, these observations suggest that Tie-1 is important for maintaining vascular integrity. In contrast, embryos lacking Tie-2 died earlier in gestation and had obviously retarded growth of the head and heart. The vasculature of Tie-2 knockout mice was abnormally developed, suggesting that Tie-2 is important for vasculogenesis. Thus, the expression of Tie receptors in embryos and adults, the effect of expression of dominant-negative receptors in transgenic mice and the effect of deletion of Tie receptors on development strongly implicate them as important RTKs in the angiogenesic process.

EGF receptor

EGF and transforming growth factor type-α (TGF-α) bind to EGF receptors with comparable affinity, but TGF-α appears to be a more potent mediator of angiogenesis in a hamster cheek pouch assay[52]. TGF-α is secreted from some tumor

cells[53] and is seen in psoriatic epidermis[54]. Thus, signaling through EGF receptors has been classified as angiogenic. In support of this classification, inhibitors of EGF or TGF-α binding have been used in experimental models to validate the pathway. For example, a fragment of EGF has been demonstrated to inhibit EGF-induced angiogenesis in a vitelline membrane assay[55]. Furthermore, neutralizing antibodies prevent TGF-α-induced tube formation by human omentum microvascular endothelial cells in collagen gels[56]. Although these observations appear to confirm that ligand-induced angiogenesis can be inhibited if the initial induction is prevented, they do not validate EGF receptors as critical signaling receptors for angiogenesis. Gross abnormalities of the vasculature have not been reported in mouse embryos lacking TGF-α (Refs 57,58) or EGF receptors[59–61], or with an EGF receptor mutation that attenuates signaling[62]. In addition, the observation that activation of EGF receptors can induce the expression of VEGF (Ref. 63) suggests that EGF and TGF-α are more likely to be indirect angiogenic factors.

FGF receptors

When a secreted form of FGF-1 (acidic FGF) was expressed in porcine arteries by *in vivo* gene transfer, it induced neointimal hyperplasia and angiogenesis within the neointima[64]. Neointimal hyperplasia was not observed in expanded polytetrafluoroethylene (ePTFE, Gore-Tex) vascular grafts coated with FGF-1 (Ref. 65), but significant enhancement of endothelialization was induced by the FGF-1 coating. In *in vitro* models of angiogenesis, blocking FGF-2 (bFGF) interaction with its receptor by antibodies to FGF-2 (Refs 66,67), platelet factor 4 (Ref. 67), or mutations of the heparin-binding site[68] resulted in inhibition of various steps involved in angiogenesis, such as induction of endothelial cell protease expression[66,68], cellular invasion[66] and formation of capillary-like tubes[67,68]. There are also correlative observations relating the expression of FGF-2 and FGFR1 to cardiac development[69,70] and endothelium re-establishment after vessel injury[71]. Dominant-negative FGF receptors have been targeted to the eye lens[72,73], epidermis[74] and lung[75], but not to endothelium. Mouse embryos homozygous for deletion of FGFR1 died early in development (before E10.5) and exhibited gross abnormalities in mesodermal patterning[76,77]. Neither group[76,77] specifically reported on the effects that FGFR1 deletion has on vasculogenesis. Thus, although FGFs and their receptors have been implicated in angiogenesis, validation of a specific role for one of the four known FGF receptors or ten known FGFs is still lacking.

HGF/SF receptor (MET)

Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, MET (HGFR), have many important roles in embryogenesis (for review, see Ref. 78). However, regulation of vasculogenesis does not appear to be one. Embryos homozygous for deletions of HGF/SF (Refs 79,80) or MET (Ref. 81) developed a normal vascular system, but died between E13.5 and E16.5 from abnormal development of the placenta and liver. In matrix and cornea models, HGF/SF-induced angiogenesis which was inhibited with anti-HGF/SF antibodies[82,83]. Analysis by RT-PCR (reverse transcriptase polymerase chain reaction) of cells infiltrating a Matrigel plug containing HGF/SF revealed the expression of several angiogenic factors and chemokines, including VEGF (Ref. 84). However, anti-VEGF antibodies were effective at only partial attenuation of HGF/SF-induced angiogenesis[84]. Thus, it is not clear whether HGF/SF-induced angiogenesis occurs through a direct or an indirect mechanism.

B61 receptor (ECK)

During embryogenesis, ECK has been implicated as having a role in pattern formation in gastrulation, hindbrain segmentation and limb development[85]. It is unknown whether ECK is expressed during development of the vascular system. In contrast, B61, a ligand for ECK, is expressed in endothelial cells of the developing vascular system and the endocardium of the developing heart, and has been postulated to have a role in vasculogenesis/angiogenesis[86]. The expression of ECK has been demonstrated in human umbilical vein endothelial cells, and B61 was shown to induce angiogenesis in a cornea model[87]. Because B61 can be induced by TNF-α (Ref. 88), which has been reported to be an angiogenic factor[89], Pandey and coworkers[87] investigated a putative role for B61 in TNF-α-mediated angiogenesis. Antibodies to B61 inhibited TNF-α-induced angiogenesis in a cornea model, indicating that B61–ECK interactions are responsible for this angiogenesis[87]. Although this clearly demonstrates that TNF-α induces angiogenesis via an indirect mechanism, it has yet to be determined if B61 stimulation of ECK represents a direct or an indirect mechanism.

PDGF receptors

Spatial and temporal expression of PDGF-BB and the PDGF β-receptor (PDGFRβ) suggests that they may play a role in angiogenesis. Both are expressed in vessels in human placenta[90], healing wounds, adenocarcinoma[91] and glioblastoma[92]. There is some discrepancy as to what cell types

express the receptor and whether the growth factor acts by an autocrine or a paracrine mechanism, although this may be tissue-dependent. There are also some contradictory studies addressing the expression patterns of the PDGF receptor in *in vitro* tube formation assays[93–95]. It is likely that PDGF is involved in angiogenesis, but, like EGF/TGF-α, it may play an indirect role by inducing VEGF (Refs 96,97). It may also exert growth-stimulatory effects on pericytes[91] and fibroblast-like cells[95,98] that surround the endothelial cells.

IGF-1 receptor

As with PDGF, insulin-like growth factor-1 (IGF-1) has been observed in angiogenic tissues, where it is known to be released by monocytes[99,100]. It also induces *in vitro* tube formation by endothelial cells[101] and stimulates the growth of fibroblast-like cells in vascular explants[98]. IGF-1 has been implicated in the angiogenesis that occurs in diabetic retinopathy because it is increased in the vitreous of patients and stimulates growth of human retinal endothelial cells[102]. Furthermore, IGF-1 induces angiogenesis in rabbit corneas[102,103]. Recently, IGF-1 was found to induce the expression of VEGF in several colon carcinoma cell lines[127]. Thus, IGF-1 may exert its angiogenic effects indirectly through VEGF.

Therapeutic strategies for inhibition of RTKs

Inhibition of angiogenesis as a possible mode of therapeutic intervention was first proposed by Folkman[104]. Advances in the understanding of the biology of angiogenesis have led to several potential modes for intervention, as illustrated in Figure 6. These include inhibition of angiogenic factors from surrounding cells, neutralization or inhibition of angiogenic factor binding to endothelial cells, signal transduction inhibition, prevention of basement membrane breakdown and inhibition of cell–cell interactions. Some of the molecules that inhibit these processes and which are in development as anti-angiogenic agents are listed in Table 1.

The role of RTKs in the formation of new blood vasculature associated with human disease has provided a strong rationale to identify ways of inhibiting the function of these enzymes. The approaches to achieve this objective have been quite varied. The vast majority of efforts have focused on the inhibition of FGF and VEGF receptors. In addition, other RTKs have been implicated in angiogenesis, but therapeutic strategies are few or lacking. Nonetheless, these targets would be amenable to many of the approaches that have been taken using the FGF and VEGF receptor targets. The therapeutic modalities that have been studied for abrogation of FGF- and VEGF-dependent signaling include the use of nucleotides (gene therapy and antisense), proteins (antibodies, receptor and ligand decoys) and low-molecular-weight compounds.

Millauer and coworkers[38,39] have shown that expression of a dominant-negative form of Flk-1 in subcutaneous implants of tumor cells from a wide variety of sources can lead to tumor growth inhibition. These findings suggest that introduction of inactive forms of Flk-1 into tumor endothelia may repress formation of new blood vasculature at the site of tumor growth. This study provides the rationale to support the use of viral and nonviral DNA transfer methods to enable expression of receptor-specific proteins resulting in inhibition of receptor function in the presence of ligand. In addition, the use of antisense oligonucleotides *in vitro* has been shown to reduce VEGF production by endothelial cells grown under conditions of oxygen depletion, leading to a block in DNA synthesis associated with VEGF-dependent proliferation of endothelial cells[105]. Furthermore, an antisense oligomer corresponding to bFGF has been shown to block proliferation of bovine aortic endothelial cells[106]. Taken together, the expression of dominant-negative and inactivating receptors or the use of antisense strategies that target local expression of ligands such as VEGF or FGF may provide a means to modulate receptor function. The limitations of the use of such techniques are more related to the pharmacological requirements for such agents in a clinical setting – namely, delivery of genes or nucleotides to the appropriate cells at the site of angiogenesis followed by a sustained effect on the process.

Several lines of evidence support the use of proteins to inhibit the angiogenic component of human disease. For instance, it has been shown that the use of anti-VEGF antibodies can result in inhibition of the growth of human tumor cells in nude mice[23]. In this case, VEGF expression was shown to be a common feature of human colorectal neoplasms. Growth of these neoplasms as subcutaneous lesions or as hepatic metastases could be substantially reduced following intraperitoneal injection of anti-VEGF antibodies. In a similar fashion, anti-VEGF antibodies have been shown to block angiogenesis of human retinal pigment epithelial cells grown *in vitro*[107]. Anti-FGF antibodies have also been shown to block *in vitro* angiogenesis[108]. In addition to antibodies directed against ligands, anti-Flk-1 antibodies[27] and soluble Flk-1 receptor decoys[109] were utilized to inhibit VEGF-induced proliferation of human umbilical vein endothelial cells.

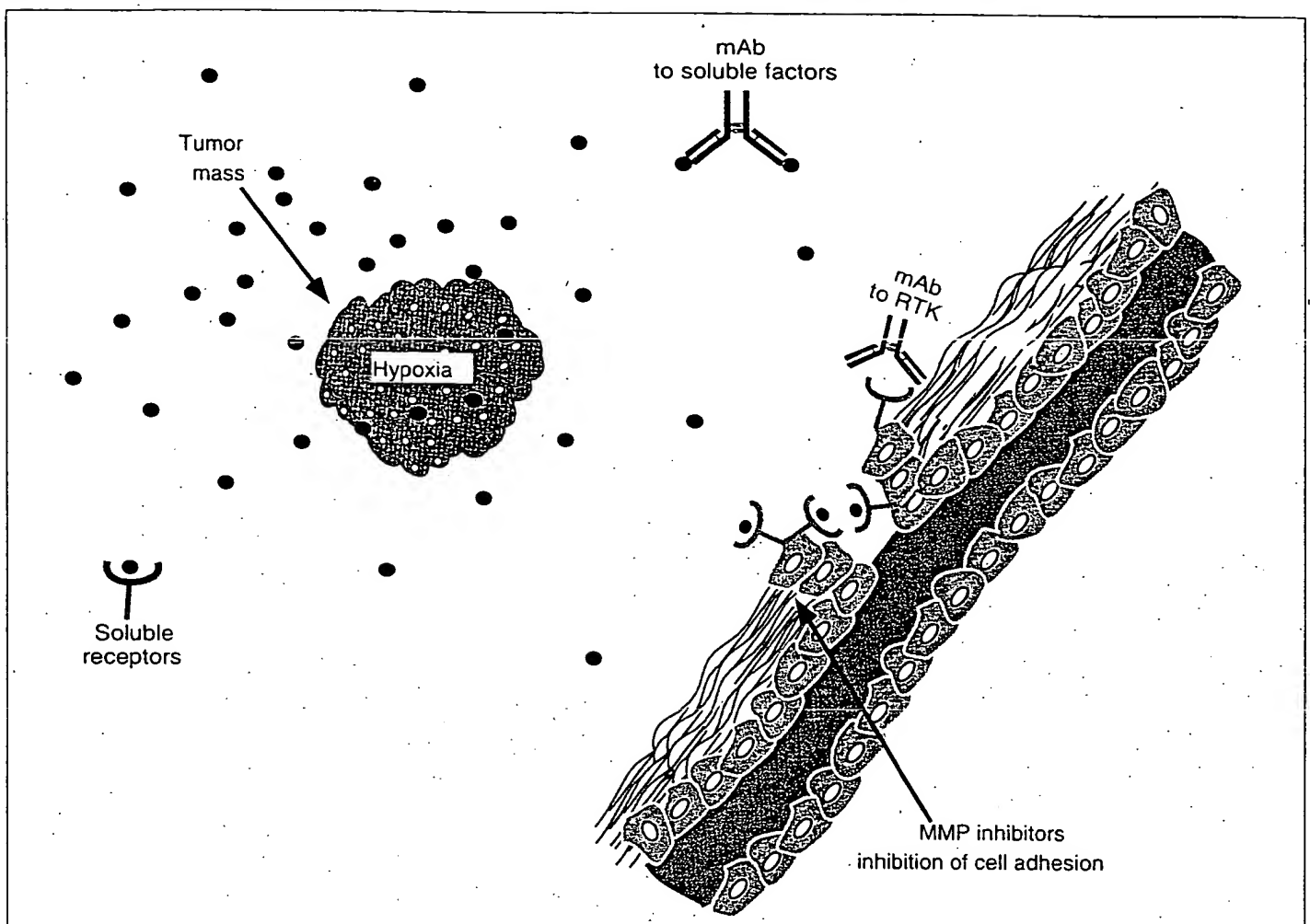

Figure 6. *Possible strategies for inhibition of angiogenesis. Because tumor angiogenesis involves several distinct processes, any one of several strategies might be successful in inhibiting the overall mechanism. These are summarized in the text; angiogenic inhibitors in development are listed in Table 1. mAb, monoclonal antibody; RTK, receptor tyrosine kinase; MMP, matrix metalloproteinase.*

The use of low-molecular-weight compounds to treat angiogenesis represents an ever-expanding area of research activities. Suramin, a nonspecific inhibitor of growth factor receptors, was shown to block bFGF-dependent proliferation of bovine aortic endothelial cells[106,110], angiogenesis in the chicken chorioallantoic membrane[106,110] and bFGF-dependent rat corneal angiogenesis[110]. Recently, inhibitors of the tyrosine kinase activities associated with Flk-1 following VEGF stimulation have been used to block VEGF-dependent proliferation of endothelial cells. For instance, genistein was shown to inhibit tyrosine phosphorylation events and cell proliferation in response to VEGF stimulation of bovine aortic endothelial cells[111]. In addition, lavendustin A blocked angiogenesis following subcutaneous implantation of VEGF-coated sponges[112]. More recently, Strawn and coworkers[113] were able to show that several distinct classes of compounds were effective at inhibiting Flk-1 tyrosine autophosphorylation in cells. In addition, some of these agents also abrogated cell signaling events that resulted in inhibition of endothelial cell proliferation following VEGF stimulation. Studies such as these provide a rationale to develop potent and selective inhibitors of Flk-1 for use as agents to inhibit angiogenic processes associated with human disease, including cancer.

Future dir ctions

Most anti-angiogenic agents are in development as anti-cancer therapeutics. Based on the fact that angiogenesis

Table 1. Selected angiogenic inhibitors in development[a]

Class of inhibitor	Chemical	*In vitro* and *in vivo* inhibitory effects	Stage of development	Company
Polypeptides				
Interferon α	Glycoprotein	EC migration; release of angiogenic factor	Clinical trials	Hoffmann-La Roche, Schering Plough
Platelet factor 4 (recombinant)	Tetrameric protein	EC proliferation; capillary formation; tumor angiogenesis	Clinical trials	Repligen
Angiostatin	Plasminogen fragment	EC proliferation; angiogenesis; metastatic growth; tumor growth	Preclinical studies	EntreMed
Bactericidal/permeability-increasing (BPI) protein derivative 23	Recombinant protein fragment	FGF-induced angiogenesis; B16 melanoma metastasis	Preclinical studies	Xoma
Humanized anti-$\alpha_v\beta_3$ antibody (LM609)	Monoclonal antibody	Cell binding to fibrinogen; tumor growth; TNF-α- and bFGF-induced angiogenesis; angiogenesis in CAM	Preclinical studies	Ixsys
Anti-VEGF monoclonal antibody	Monoclonal antibody	VEGF-induced angiogenesis *in vitro* and *in vivo*; tumor growth and metastasis	Preclinical studies	Genentech
Anti-Flk-1 monoclonal antibody (DC101)	Monoclonal antibody	VEGF binding to Flk-1; VEGF-induced phosphorylation of Flk-1; tumor growth	Preclinical studies	ImClone Systems
Soluble Flt-1 receptor	Receptor fragment	Receptor binding to VEGF and PlGF; VEGF-induced EC mitogenesis	Preclinical studies	Merck
Carbohydrates				
Tecogalan (DS4152)	Sulfated polysaccharide–peptidoglycan complex	bFGF binding to EC; EC proliferation; angiogenesis in CAM and tumors; tumor growth	Clinical trials	Daiichi
bFGF carbohydrate inhibitor (GM1474)	Sulfated carbohydrate	bFGF-induced EC proliferation; tumor growth; metastases in B16 model	Preclinical studies	Glycomed, Ligand
Glyceptor mimetic inhibitor of bFGF (GL14.2)	Carbohydrate	Binding of bFGF or VEGF to cell surface glycosaminoglycan; tumor growth	Preclinical studies	Glycan, ProsCure
Antibiotics				
AGM1470 (TNP 470)	Fumagillin analog	Expression of cyclins and activation of cyclin-dependent kinases; EC migration and proliferation; collagenases; tumor angiogenesis and growth	Clinical trials	Takeda, Abbott
Polycations and polyanions				
Suramin	Polyanionic compound	Binding of bFGF to receptor; tumor growth and angiogenesis	Clinical trials	Warner-Lambert/NIH
Small molecules				
Inhibitors of Flk-1	Small-molecule inhibitors	Flk-1 phosphorylation; VEGF-induced EC proliferation; angiogenesis in CAM; tumor growth, angiogenesis, and metastasis	Preclinical studies	SUGEN
Inhibitors of VEGF-Flt binding	Small molecule inhibitors	VEGF binding; VEGF-induced calcium changes and proliferation in ECs; VEGF-induced vascular permeability	Preclinical studies	Texas Biotechnology

Inhibitors of phosphatidic acid	Small-molecule inhibitors	FGF-, VEGF- and PDGF-induced SMC and EC mitogenesis; VEGF-induced EC migration in Matrigel	Preclinical studies	Cell Therapeutics
Thalidomide/analogs	Polycyclic teratogen	EC responses; TNF-α formation; bFGF-induced corneal angiogenesis	Clinical trials	EntreMed, Bristol-Myers Squibb
Batimastat/Marimastat	Small-molecule inhibitors	Matrix metalloproteinases; hemangioma growth; EC invasion; *in vivo* angiogenesis	Clinical trials	British Biotech
Urokinase receptor antagonists	Small-molecule inhibitors	Plasminogen activation; EC capillary tube formation; corneal and *in vivo* Matrigel angiogenesis; tumor growth and angiogenesis	Preclinical studies	Chiron
Oligonucleotides				
VEGF antisense oligonucleotide	Oligonucleotides	Expression of VEGF in tumor cells; ischemia-induced retinal neovascularization	Preclinical studies	Hybridon
Ribozymes targeting VEGF receptors	Nucleotides	VEGF-stimulated growth of human microvascular ECs and corneal angiogenesis	Preclinical studies	Ribozyme Pharmaceuticals

[a]bFGF, basic fibroblast growth factor; CAM, chorio-allantoic membrane; EC, endothelial cell; Flk-1, vascular endothelial cell factor receptor-2 (VEGFR2); Flt-1, vascular endothelial cell factor receptor-1 (VEGFR1); PDGF, platelet-derived growth factor; PlGF, placental growth factor; SMC, smooth muscle cell; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

inhibition not only blocks primary tumor growth but also reduces metastasis to distant sites[114], inhibitors may be useful in first-line therapies as well as in an adjunctive situation. Other diseases are also likely to benefit from this type of therapy. For example, abnormalities of microvessels in the papillary dermis are thought to be important in sustaining epidermal hyperproliferation in dermatological disorders such as psoriasis and scleroderma[115,116]. Biopsies of human tissues from psoriatic lesions and contact dermatitis have been shown to have increased levels of VEGF and Flk-1 (Refs 117,118). In addition, the receptors for VEGF, Flk-1 and Flt-1 were found to be overexpressed in papillary dermal microvascular endothelial cells[117].

Retinal neovascularization, the final common pathway leading to vision loss in diseases such as retinopathy of prematurity, age-related macular degeneration and diabetic retinopathy, is another potential therapeutic area for angiogenesis inhibitors. VEGF is present in ocular fluid of patients with diabetic retinopathy and other retinal disorders[119]. In animal models, elevated intraocular levels of VEGF are associated with active retinal neovascularization, and decreased levels parallel the regression in proliferative retinopathy[120,121]. The potential use of Flk-1 inhibitors as specific therapy for ischemic retinal disease is suggested by significant inhibition of retinal neovascularization observed with antisense VEGF and soluble VEGF receptor chimeric proteins[109].

Rheumatoid arthritis, although characterized by inflammation and immunoproliferation, is another disease in which angiogenesis has been implicated in the disease process. Growth of the pannus, which contributes to the destruction of joint cartilage, is thought to be dependent on local angiogenesis. Several investigators have shown the expression of VEGF in synovial fluid, subsynovial macrophages, fibroblasts surrounding microvessels in the pannus, vascular smooth muscle cells and synovial lining cells from patients with rheumatoid arthritis[122–124]. In atherosclerosis, the development of the atherosclerotic plaque is associated with neovascularization in the thickened intima and media of vascular walls. Although the mechanism and stimulus for neovascularization are unknown, the plaques have been shown to have angiogenic activity, as measured by the ability to induce growth of new vessels in a rabbit cornea model[125]. Also, conditioned medium from smooth muscle cells stimulated endothelial cell proliferation[126], and a neutralizing antibody to VEGF was able to attenuate the angiogenic activity.

It is apparent that anti-angiogenic agents will potentially have broad applications in the clinic. While there are several strategies for inhibiting angiogenesis, the data generated from many laboratories suggest that targeting receptors for angiogenic growth factors will lead to new treatments. As discussed previously, there are several approaches to disrupting the signaling of receptor tyrosine kinases, including

the use of antibodies or receptor decoys to block ligand binding, and reducing the function of the receptor using antisense or dominant-negative technology. Additionally, because RTKs represent proteins with enzymatic function, they lend themselves to pharmacological intervention with small-molecule compounds. Irrespective of the mechanism for inhibition, it is likely that these agents will be used for chronic therapy in most diseases. This will therefore necessitate the development of drugs with a good safety profile and a convenient route of administration.

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The Protein Kinase Family: Conserved Features and Deduced Phylogeny of the Catalytic Domains

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In recent years, members of the protein kinase family have been discovered at an accelerated pace. Most were first described, not through the traditional biochemical approach of protein purification and enzyme assay, but as putative protein kinase amino acid sequences deduced from the nucleotide sequences of molecularly cloned genes or complementary DNAs. Phylogenetic mapping of the conserved protein kinase catalytic domains can serve as a useful first step in the functional characterization of these newly identified family members.

THE PROTEIN KINASES ARE A LARGE FAMILY OF ENZYMES, many of which mediate the response of eukaryotic cells to external stimuli (*1, 2*). The number of unique members of the protein kinase family that have been described has recently risen exponentially (*3*) and now approaches 100. The surge in the number of known protein kinases has been largely due to the advent of gene cloning and sequencing techniques. Amino acid sequences deduced from nucleotide sequences are considered to represent protein kinases if they include certain key residues that are highly conserved in the protein kinase "catalytic domain."

Two different molecular approaches have been most instrumental in the isolation of novel protein kinase–encoding genes or cDNAs: (i) complementation or suppression of genetic defects in invertebrate regulatory mutants, and (ii) screening DNA libraries by using protein kinase genes as hybridization probes under low stringency conditions. Recently, an approach that uses degenerate oligonucleotides as probes has led to the identification of several novel putative protein kinase genes and cDNAs (*4, 5*). The oligonucleotide probes are designed to recognize target sequences that encode short amino acid stretches highly conserved in protein kinase catalytic domains.

In this article, we present an alignment of catalytic domain amino acid sequences from 65 different members of the protein kinase family, including many putative protein kinase sequences recently deduced from nucleotide sequence data. Based on this alignment, we first identify and discuss conserved features of the catalytic domains and then provide a visual display of the various intersequence relations through construction of a catalytic domain phylogenetic tree. Catalytic domains from protein kinases having similar modes of regulation or substrate specificities are found to cluster together within the tree. This clustering would appear to be of predictive value in the determination of the properties and function of novel protein kinases.

Catalytic Domain Amino Acid Sequences

Protein kinase catalytic domains range from 250 to 300 amino acid residues, corresponding to about 30 kD. Fairly precise boundaries for the catalytic domains have been defined through an analysis of conserved sequences (*1, 6*, see below) as well as by assay of truncated enzymes (*7, 8*). The location of the catalytic domain within the protein is not fixed but, in most single subunit enzymes it lies near the carboxyl terminus, the amino terminus being devoted to a regulatory role. In protein kinases having a multiple subunit structure, subunit polypeptides consisting almost entirely of catalytic domain are common. All protein kinases thus far characterized with regard to substrate specificity fall within one of two broad classes, serine/threonine–specific and tyrosine-specific. Although both classes of protein kinase have very similar catalytic domain primary structures, certain short amino acid stretches appear to characterize each class (*4*), and these regions can be used to predict whether a putative protein kinase will phosphorylate tyrosine or serine/threonine.

Members of the protein–serine/threonine kinase and protein-

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tyrosine kinase families with reported catalytic domain amino acid sequences are listed in Tables 1 and 2, respectively. They are classified within the tables according to similarities in primary structure, based on deduced catalytic domain phylogeny. Included in the tables are all confirmed and putative protein kinases for which the catalytic domain sequence was available as of November 1987 (*9*). Presumed functional homologs from different vertebrate species are listed together. Presumed invertebrate functional homologs of protein kinases also found in vertebrates, however, are given separate listings as a reflection of greater evolutionary distance and the possibility of functional divergence. The asterisks indicate protein kinases that have catalytic domains that are included in the amino acid sequence alignment. We will use the abbreviated names from the tables to refer to individual protein kinases.

Of the 45 unique vertebrate protein kinase family members included in Tables 1 and 2, 22 are serine/threonine–specific and 23 are tyrosine-specific. Fourteen of the vertebrate protein–serine/threonine kinases fall within one of the three subgroups that can be

Table 1. Protein–serine/threonine kinase family members.

A. Cyclic nucleotide–dependent subfamily
- cAPK-α: cAMP-dependent protein kinase catalytic subunit, α form
  - *-bovine cardiac muscle protein (*26*)
  - -mouse S49 lymphoma cell cDNA (*35*)
- cAPK-β: cAMP-dependent protein kinase catalytic subunit, β form
  - *-bovine pituitary cDNA (*36*)
  - -mouse S49 lymphoma cell cDNA (*37*)
- SRA3: cAMP-dependent protein kinase from yeast, RAS suppressor
  - *-*Saccharomyces cerevisiae* genomic DNA (*38*)
- TPK1(PK25): cAMP-dependent protein kinase from yeast, type 1
  - *-*S. cerevisiae* genomic DNA (*39, 40*)
- TPK2: cAMP-dependent protein kinase from yeast, type 2
  - *-*S. cerevisiae* genomic DNA (*39*)
- TPK3: cAMP-dependent protein kinase from yeast, type 3
  - *-*S. cerevisiae* genomic DNA (*39*)
- cGPK: guanosine 3′,5′-monophosphate (cGMP)–dependent protein kinase
  - *-bovine lung protein (*41*)

B. Calcium-phospholipid–dependent subfamily
- PKC-α: protein kinase C, α form
  - *-bovine brain cDNA (*42*)
  - -rabbit brain cDNA (*43*)
  - -human brain cDNA (partial) (*44*)
- PKC-β: protein kinase C, β form
  - *-bovine brain cDNA (*44*)
  - -rat brain cDNA (two splice forms) (*45, 46*)
  - -rabbit brain cDNA (two splice forms) (*43*)
  - -human brain cDNA (*44*)
- PKC-γ: protein kinase C, γ form
  - *-bovine brain cDNA (*44*)
  - -rat brain cDNA (*45*)
  - -human brain cDNA (*44*)
- PKC-ε: protein kinase C, ε form
  - -rat brain cDNA (RP16 clone) (partial) (*46*)
- DPKC: *Drosophila* gene product related to protein kinase C
  - *-*D. melanogaster* cDNA (*47*)

C. Calcium-calmodulin–dependent subfamily
- CaMII-α: calcium-calmodulin–dependent protein kinase type II, α subunit
  - *-rat brain cDNA (*48*)
- CaMII-β: calcium-calmodulin–dependent protein kinase type II, β subunit
  - *-rat brain cDNA (*49*)
- PhK-γ: phosphorylase kinase, γ subunit
  - *-rabbit skeletal muscle protein and cDNA (*50*)
  - -mouse muscle cDNA (*51*)
- MLCK-K: myosin light chain kinase, skeletal muscle
  - *-rabbit skeletal muscle protein (*52*)
- MLCK-M: myosin light chain kinase, smooth muscle
  - *-chicken gizzard cDNA (*53*)
- PSK-H1: putative protein-serine kinase
  - *-human HeLa cell cDNA (*4, 54*)
- PSK-C3: putative protein-serine kinase
  - -human HeLa cell cDNA (partial) (*4*)

D. SNF1 subfamily
- SNF1: "sucrose nonfermenting" mutant wild-type gene product
  - *-*S. cerevisiae* genomic DNA (*55*)
- nim1$^+$: "new inducer of mitosis"; suppressor of cdc25 mutants
  - *-*Schizosaccharomyces pombe* genomic DNA (*56*)
- KIN1: putative yeast protein kinase
  - *-*Saccharomyces cerevisiae* genomic DNA (*5*)
- KIN2: putative yeast protein kinase related to KIN1
  - *-*S. cerevisiae* genomic DNA (*5*)

E. CDC28-cdc2$^+$ subfamily
- CDC28: "cell-division-cycle" gene product in yeast
  - *-*S. cerevisiae* genomic DNA (*57*)
- cdc2$^+$: "cell-division-cycle" gene product in yeast
  - *-*Schizosaccharomyces pombe* genomic DNA (*58*)
- CDC2Hs: human functional homolog of cdc2$^+$
  - *-human transformed cell line cDNA (*33*)
- PSK-J3: putative protein kinase related to CDC28-cdc2$^+$
  - *-human HeLa cell cDNA (*4, 59*)
- KIN28: putative protein kinase related to CDC28-cdc2$^+$
  - *-*Saccharomyces cerevisiae* genomic DNA (*60*)

F. Casein kinase subfamily
- CKIIα: casein kinase II, α subunit
  - -bovine lung protein (partial) (*61*)
- DCKII: *Drosophila* casein kinase II, α subunit
  - *-*D. melanogaster* cDNA (*62*)

G. Raf-Mos proto-oncogene subfamily
- Raf: cellular homolog of oncogene products from 3611 murine sarcoma virus and Mill Hill 2 avian acute leukemia virus
  - *-human fetal liver cDNA (*63*)
- A-Raf: cellular oncogene product closely related to Raf
  - *-human T cell cDNA (*64*)
  - -mouse spleen cDNA (*65*)
- PKS: cellular gene product closely related to Raf
  - *-human fetal liver cDNA (*66*)
- Mos: cellular homolog of oncogene product from Moloney murine sarcoma virus
  - *-human placenta genomic DNA (*67*)
  - -mouse NIH 3T3 cell genomic DNA (*68*)
  - -rat 3Y1 cell genomic DNA (*69*)

H. STE7 subfamily
- STE7: "sterile" mutant wild-type allele gene product
  - *-*S. cerevisiae* genomic DNA (*70*)
- PBS2: polymixin B antibiotic resistance gene product
  - *-*S. cerevisiae* genomic DNA (*71*)

I. Family members with no close relatives
- CDC7: "cell-division-cycle" gene product
  - *-*S. cerevisiae* genomic DNA (*72*)
- wee1$^+$: "reduced size at division" mutant wild-type gene product
  - *-*Schizosaccharomyces pombe* genomic DNA (*73*)
- ran1$^+$: "meiotic bypass" mutant wild-type allele gene product
  - *-*S. pombe* genomic DNA (*74*)
- PIM-1: putative transforming protein induced by murine leukemia virus integration
  - *-mouse BALB/c cell genomic DNA (*75*)
- HSVK: herpes simplex virus-US3 gene product
  - *-herpes simplex virus genomic DNA (*76*)

*Protein kinases that have catalytic domains included in the amino acid sequence alignment.

classified according to their mode of regulation: cyclic nucleotide–dependent, calcium-phospholipid–dependent, and calcium-calmodulin–dependent. Two of the serine/threonine kinases, Mos and Raf (products of the c-*mos* and c-*raf* genes, respectively), are cellular homologs of transforming proteins encoded by the retroviral oncogenes. Other members of the serine/threonine group with demonstrated oncogenic potential are A-Raf (a distinct Raf-related member), and PIM-1 (a putative transforming protein activated by viral integration). Three vertebrate serine/threonine kinases (CDC2Hs, PSK-J3, and CKIIα) are closely related, by various degrees, to the yeast cell cycle control protein kinases CDC28 and $cdc2^+$. A protein–serine/threonine kinase has been described in herpes simplex virus (HSVK) and, like the retroviral oncogenes, probably originated as a eukaryotic cellular sequence. The protein-tyrosine kinases can be further grouped as members of either the Src subfamily or one of three different growth factor receptor subfamilies. The protein-tyrosine kinases encoded by the c-*abl* and c-*fes*/*fps* genes may be considered distant members of the Src subfamily. At least nine of the protein-tyrosine kinase genes have been transduced by retroviruses where they encode transforming proteins.

Twenty-five additional sequences listed in Tables 1 and 2 derive from invertebrate species. Eight are from *Drosophila*, one from nematode, and the other 16 are from the budding or fission yeasts. Many of the *Drosophila* protein kinases, as well as the nematode protein kinase, were identified by screening DNA libraries with probes from a vertebrate protein kinase gene or cDNA and thus are likely to represent functional homologs of the vertebrate enzymes. The *Drosophila* "sevenless" (7less) protein kinase and most of the yeast protein kinases were identified through molecular genetics. All of the yeast protein kinases identified to date fall within the serine/threonine–specific class, despite directed attempts to identify protein-tyrosine kinases in yeast (*5*). This observation, together with the fact that many of the protein-tyrosine kinase catalytic domains are components of growth factor receptor molecules, suggests that tyrosine specificity may have been a recent development in catalytic domain evolution, arising in conjunction with the acquisition of multicellularity and serving a role in cell-cell communication.

Table 2. Protein-tyrosine kinase family members.

A. Src subfamily
- Src: cellular homolog of oncogene product from Rous avian sarcoma virus
  - *-human fetal liver genomic DNA (*77*)
  - -mouse brain cDNA; neuronal alternate splice form (*78*)
  - -chicken genomic DNA (*79*)
  - -*Xenopus laevis* ovary cDNA (partial) (*80*)
- Yes: cellular homolog of oncogene product from Yamaguchi 73 avian sarcoma virus
  - *-human embryo fibroblast cDNA (*81*)
- Fgr: cellular homolog of oncogene product from Gardner-Rasheed feline sarcoma virus
  - *-human genomic DNA (*82*)
  - -human B lymphocyte cell line cDNA (amino terminus) (*83*)
- FYN: putative protein-tyrosine kinase related to Fgr and Yes
  - *-human fibroblast cDNA (*84*)
- LYN: putative protein-tyrosine kinase related to LCK and Yes
  - *-human placenta cDNA (*85*)
- LCK: lymphoid cell protein-tyrosine kinase
  - *-human (JURKAT) T cell leukemia line cDNA (*86*)
  - -mouse (LSTRA) T cell lymphoma line cDNA (*87*)
- HCK: hematopoietic cell putative protein-tyrosine kinase
  - *-human placenta and peripheral leukocyte cDNAs (*88*)
- Dsrc64: *Drosophila* gene product related to Src; polytene locus 64B
  - *-*D. melanogaster* genomic DNA (*89*, *90*)
- Dsrc28: *Drosophila* gene product related to Src; polytene locus 28C
  - *-*D. melanogaster* adult female cDNA (*91*)

B. Abl subfamily
- Abl: cellular homolog of oncogene product from Abelson murine leukemia virus
  - *-human fetal liver cDNA (*92*)
- ARG: putative protein-tyrosine kinase related to Abl
  - -human genomic DNA (partial) (*93*)
- Dash: *Drosophila* gene product related to Abl
  - *-*D. melanogaster* genomic DNA (*90*)
- Nabl: nematode gene product related to Abl
  - *-*Caenorhabditis elegans* genomic DNA (*94*)
- Fes/Fps: cellular homolog of oncogene products from Gardner-Arnstein and Snyder-Theilen feline sarcoma viruses and Fujinami and PRCII avian sarcoma viruses
  - *-human genomic DNA (*95*)
  - -feline genomic DNA (*96*)
  - -chicken genomic DNA (*97*)

C. Epidermal growth factor receptor subfamily
- EGFR: epidermal growth factor receptor; cellular homolog of oncogene product (v-Erb-B) from AEV-H avian erythroblastosis virus
  - *-human placenta and A431 cell line cDNAs (*98*)
- NEU: cellular oncogene product activated in induced rat neuroblastomas (also called ERB-B2 or HER2)
  - *-human placenta and gastric cancer cell line cDNAs (*99*)
  - -rat neuroblastoma cell line cDNA (*100*)
- DER: *Drosophila* gene product related to EGFR
  - *-*D. melanogaster* genomic DNA (*101*)

D. Insulin receptor subfamily
- INS.R: insulin receptor
  - *-human placenta cDNA (*102*)
- IGF1R: insulin-like growth factor 1 receptor
  - *-human placenta cDNA (*103*)
- DILR: *Drosophila* gene product related to INS.R
  - *-*D. melanogaster* embryo cDNA (*104*)
- Ros: cellular homolog of oncogene product from UR2 avian sarcoma virus
  - *-human placenta genomic DNA (*105*)
  - -chicken genomic DNA (*106*), chicken kidney cDNA (*107*)
- 7less: *Drosophila sevenless* gene product essential for R7 photoreceptor cell development
  - *-*D. melanogaster* eye imaginal disc cDNA (*108*)
- TRK: colon carcinoma oncogene product activated by genetic recombination
  - *-human tumor cell cDNA (*109*)
- MET: *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)–induced oncogene product
  - *-human HOS cell line cDNA (*110*)

E. Platelet-derived growth factor receptor subfamily
- PDGFR: platelet-derived growth factor receptor
  - *-mouse NR6 fibroblast cell line cDNA (*111*)
- CSF1R: colony-stimulating factor–type 1 receptor; cellular homolog of oncogene product (v-Fms) from McDonough feline sarcoma virus
  - *-human placenta cDNA (*112*)
- Kit: cellular homolog of oncogene product from Hardy-Zuckerman 4 feline sarcoma virus
  - *-human placenta cDNA (*113*)
- RET: cellular oncogene product activated by recombination
  - *-human T cell lymphoma cDNA (*114*)

F. Other receptor-like protein-tyrosine kinases
- TKR11: putative protein-tyrosine kinase
  - -chicken genomic DNA (partial) (*115*)
- TKR16: putative protein-tyrosine kinase
  - -chicken genomic DNA (partial) (*115*)

*Protein kinases that have catalytic domains included in the amino acid sequence alignment.

Conserved Features of the Catalytic Domains

To compare primary structures, we have aligned catalytic domains from the 65 protein kinases marked by an asterisk in Tables 1 and 2 (Fig. 1). The 65 sequences represent each of the separate entries in the Tables except for six family members that are not included because their catalytic domain sequences have been only partially determined. The alignment was made by eye and is parsimonious in nature; the amount of gapping introduced into the sequences in order to optimize positional similarities was kept to a minimum. The alignment clearly demonstrates the overall similarity among the catalytic domains. The catalytic domains are not conserved uniformly but, rather, consist of alternating regions of high and low conservation. Eleven major conserved subdomains are evident (Fig. 1, I to XI), separated by regions of lower conservation wherein fall the larger gaps or inserts. Very large inserts (in excess of 60 residues) occur in CDC7 between subdomains VII and VIII and between subdomains X and XI, and in PDGFR, CSF1R, and Kit between subdomains V and VI. A similarity profile of the aligned catalytic domains provides a ready visualization of the subdomain structure (Fig. 2). Such an arrangement of alternating regions of high and low conservation is a common feature of homologous globular proteins (*10*) and gives some clues to higher order structure. The conserved subdomains must be important for catalytic function, either directly as components of the active site or indirectly by contributing to the formation of the active site through constraints imposed on secondary structure. The nonconserved regions, on the other hand, are likely to occur in loop structures, where folding allows the essential conserved regions to come together.

Highly conserved individual amino acids within the catalytic domains are expected to play important roles in catalysis. We will refer to amino acid positions using the residue numbering for bovine adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase catalytic subunit, α form (cAPK-α, Fig. 1). Nine positions in the alignment contain the identical amino acid residue in each of the 65 sequences. These invariant residues correspond to cAPK-α: Gly^{52}, Lys^{72}, Glu^{91}, Asp^{166}, Asn^{171}, Asp^{184}, Gly^{186}, Glu^{208}, and Arg^{280}. An additional five positions contain the identical amino acid in all but one of the sequences: Gly^{50}, Val^{157}, Phe^{185}, Asp^{220}, and Gly^{225}. Many of these most highly conserved residues directly participate in adenosine triphosphate (ATP) binding and phosphotransfer.

The consensus Gly-X-Gly-X-X-Gly, found in many nucleotide binding proteins in addition to the protein kinases (*11*), is found in subdomain I, very near the catalytic domain amino terminus. The invariant or nearly invariant residues corresponding to cAPK-α Gly^{50} and Gly^{52} fall within this consensus. Only two positions on the amino-terminal side of this consensus show conservation throughout the protein kinase family; hydrophobic residues occupy positions one and seven upstream from the first glycine in the consensus. The amino terminus of some catalytic domain polypeptides lies as close as ten residues from the first conserved glycine. A model for the ATP-binding site of v-Src (*12*), based on the three-dimensional structures from other nucleotide binding proteins, shows the Gly-X-Gly-X-X-Gly residues forming an elbow around the nucleotide, with the first glycine in contact with the ribose moiety and the second glycine lying near the terminal pyrophosphate. A nearly invariant valine residue lies within subdomain I, located just two positions on the carboxyl-terminal side of the Gly-X-Gly-X-X-Gly consensus (Val^{57} for cAPK-α) and may contribute to the positioning of the conserved glycines.

In subdomain II lies an invariant lysine, corresponding to cAPK-α Lys^{72}, that is certainly the best characterized catalytic domain residue. This lysine appears to be directly involved in the phosphotransfer reaction, possibly mediating proton transfer (*13*). In cAPK-α (*14*), v-Src (*15*), and EGFR (*16*), Lys^{72} or its equivalent reacts with the ATP analog *p*-fluorosulfonyl 5′-benzoyl adenosine, thereby inhibiting enzyme activity. Site-directed mutagenesis techniques have been used to substitute alternate amino acids at this position in v-Src (*13, 17*), v-Mos (*18*), v-Fps (*19*), EGFR (*20*), INS.R (*21*), and PDGFR (*22*). All substitutions, including arginine, result in loss of protein kinase activity. In all but three of the aligned sequences, an alanine is present two positions on the amino-terminal side of the invariant lysine in subdomain II. The invariant lysine lies 14 to 23 residues downstream of the last conserved glycine in subdomain I, but no mutations have been made to test whether this spacing is critical.

The central core of the catalytic domain, the region with greatest frequency of highly conserved residues, consists of subdomains VI through IX. The invariant or nearly invariant residues in subdomain VI (corresponding to Asp^{166} and Asn^{171}) and subdomain VII (corresponding to Asp^{184}, Phe^{185}, and Gly^{186}) also have been implicated in ATP binding. These residues are part of a feature found in a number of bacterial phosphotransferases that use ATP as phosphate donor (*23*). The aspartic acid residues corresponding to cAPK-α Asp^{166} and Asp^{184} may interact with the phosphate groups of ATP through Mg^{2+} salt bridges (*23*). The triplet corresponding to Asp^{184}-Phe^{185}-Gly^{186} in subdomain VII is of further interest in that it represents the most highly conserved short stretch in the catalytic domains. It is flanked for two positions on either side by hydrophobic or near-neutral residues.

Subdomain VIII contains the consensus triplet Ala-Pro-Glu, a conserved feature often mentioned as a key protein kinase catalytic domain indicator (*1*). The invariant residue corresponding to cAPK-α Glu^{208} contributes to the Ala-Pro-Glu consensus. In addition to the conservation of these residues, several other lines of evidence implicate this region as important in catalysis. Mutagenesis studies have shown that each residue in the Ala-Pro-Glu consensus is required for activity of v-Src (*24*). Other studies have provided evidence that this consensus lies very near the catalytic site. An affinity peptide substrate analog reacts with cAPK-α Cys^{199}, thereby inhibiting enzyme activity (*25*). Also, sites of autophosphorylation found in many protein-tyrosine kinases (*1*) as well as cAMP-dependent protein kinase [Thr^{197} (*26*)] lie within 20 residues upstream of the Ala-Pro-Glu consensus. The role of this autophosphorylation site is not entirely settled, but for several protein-tyrosine kinases there is evidence that phosphorylation of this site leads to increased catalytic activity (*27*). Autophosphorylation may result in a conformational change that allows better access of exogenous substrates to the active site.

Subdomains VI and VIII are of additional interest in that they contain residues that are specifically conserved in either the protein–serine/threonine or the protein-tyrosine kinases and, as such, may play a role in recognition of the correct hydroxyamino acid. The most striking indicator of amino acid specificity is found in subdomain VI, lying between the invariant residues corresponding to cAPK-α Asp^{166} and Asn^{171}; two of the residues implicated in ATP binding. The consensus Asp-Leu-Lys-Pro-Glu-Asn in this region is a strong indicator of serine/threonine specificity, whereas the protein-tyrosine kinase consensus is either Asp-Leu-Arg-Ala-Ala-Asn (for the vertebrate members of the Src subfamily) or Asp-Leu-Ala-Ala-Arg-Asn (for all others). Another such region is found in subdomain VIII and lies immediately on the amino-terminal side of the Ala-Pro-Glu consensus. This region is highly conserved among the protein-tyrosine kinases with a more limited conservation among the protein–serine/threonine kinases. The protein-tyrosine kinase consensus through this region is Pro-Ile/Val-Lys/Arg-Trp-Thr/Met-Ala-Pro-Glu while the protein–serine/threonine kinase

consensus is Gly-Thr/Ser-X-X-Tyr/Phe-X-Ala-Pro-Glu. These regions in subdomains VI and VIII that indicate substrate specificity have been targeted for the design of degenerate oligonucleotide probes for use in screening cDNA libraries to identify novel members of both the protein–serine/threonine (*4*) and protein-tyrosine (*28*) kinase families.

To date, no evidence has been reported concerning the possible functions of residues in conserved subdomains III, IV, V, IX, X, and XI. Subdomain IX contains a very well conserved short stretch that includes the nearly invariant residues corresponding to Asp^{220} and Gly^{225}. Subdomains III and XI each contain an invariant residue, corresponding to Glu^{91} and Arg^{280}. The latter or its equivalent must lie very near the catalytic domain carboxyl terminus. Arginine residues occupying this position reside just 16 residues upstream from both the CDC28 and HSVK polypeptide carboxyl termini, and just 19 residues upstream from both the Mos and Fes carboxyl termini. Deletion analysis of v-Src places the carboxyl terminus of the catalytic domain of the protein-tyrosine kinases at a conserved hydrophobic residue ten residues downstream of this arginine (*8*). The point mutation conferring temperature sensitivity in some *cdc28* mutants replaces this conserved arginine with glutamine (*29*).

A leap in our understanding of the functional roles of the conserved catalytic domain residues will come with the solution of a crystal structure for one of the protein kinase catalytic domains. The similarities in primary strucure should carry over to the higher order structure and catalytic mechanism as well. Other investigators have been making progress toward the solution of the three-dimensional structure of cAPK-α (*30*).

Catalytic Domain Phylogeny

Amino acid sequence alignments can be used to deduce phylogenetic relationships (*31*). We have used the alignment data from Fig. 1 to construct a phylogenetic tree of the protein kinase catalytic domains (Fig. 3). All 65 of the sequences in the alignment are included in the tree. They derive from both vertebrate and invertebrate sources and, in some cases, presumed functional homologs from both vertebrate and invertebrate sources are represented. The tree, therefore, reflects catalytic domain evolution stemming from gene duplication events (for example, when the vertebrate, mostly human, sequences are compared), speciation events (when vertebrate and invertebrate functional homologs are compared), or both.

The tree reveals a relation between catalytic domain sequence and certain biochemical properties; catalytic domains from protein kinases having similar modes of regulation or substrate specificities tend also to have similar primary structures and cluster together within the tree. Five major branch clusters are present in the tree: (i) protein-tyrosine kinases, (ii) cyclic nucleotide– and calcium-phospholipid–dependent protein kinases, (iii) calcium-calmodulin–dependent protein kinases, (iv) protein kinases closely related to SNF1, and (v) protein kinases closely related to CDC28. These major clusters account for all but 12 of the 65 sequences included in the tree. Generally, a sequence found within one of these clusters shares in excess of 35% identical amino acids with each of the other sequences in the cluster, whereas the catalytic domain sequences that do not map within the same cluster have identities in the range of 20 to 25%.

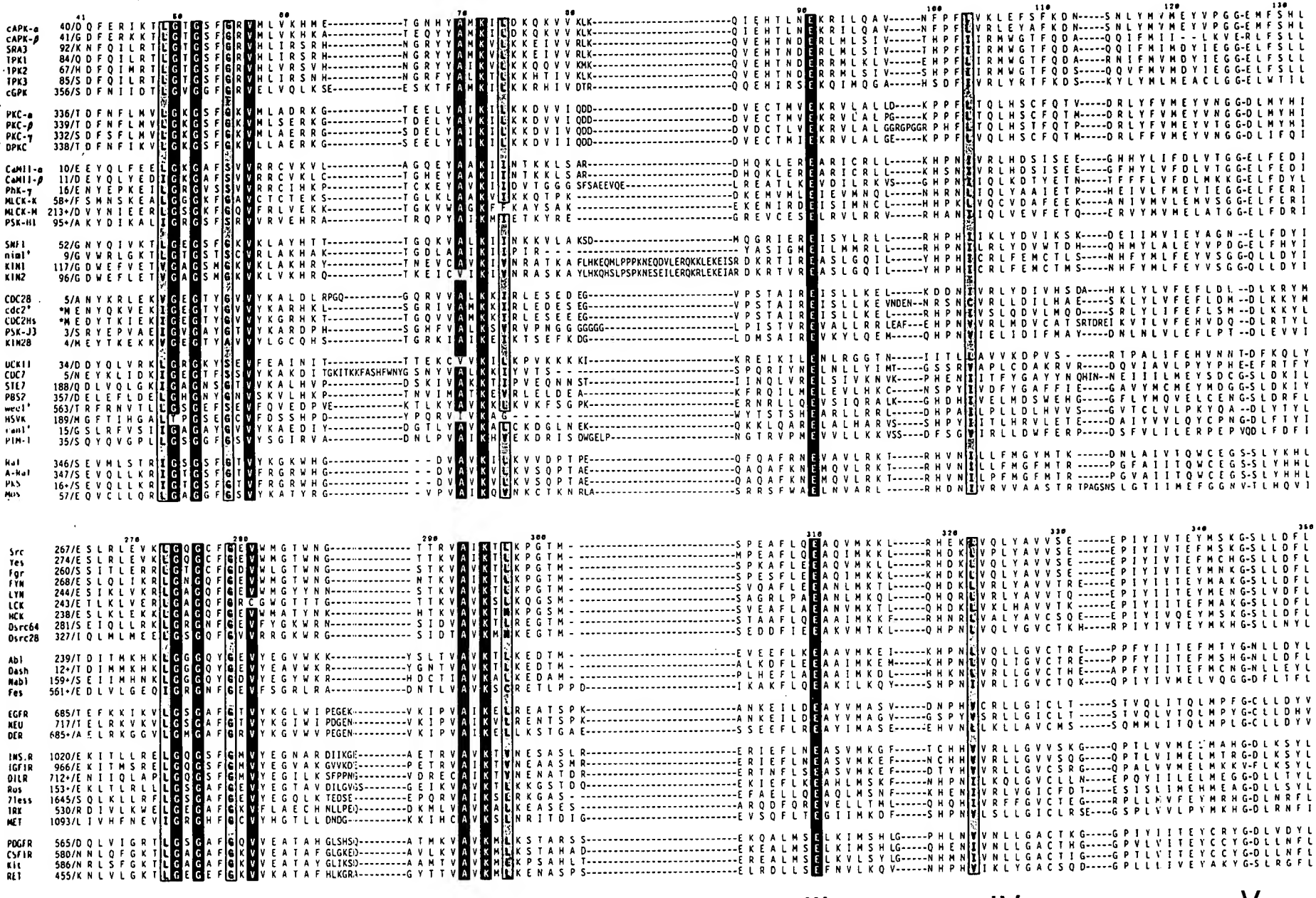
The most highly populated cluster contains all 27 confirmed or putative protein-tyrosine kinases. The large number of protein-tyrosine kinases probably reflects the intense research effort devoted to this group, rather than a true indication of their abundance relative to the protein–serine/threonine kinases. Branches leading to the Src subfamily and to each of the three receptor subfamilies diverge from the main line at about the same point. In light of the oncogenic potential of many of the protein-tyrosine kinases, it is of interest that the protein–serine/threonine kinases having the least divergence from this group include Raf and Mos, cellular homologs of retroviral oncogene products. However, another potentially oncogenic protein–serine/threonine kinase, PIM-1, is not closely related to the protein-tyrosine kinases.

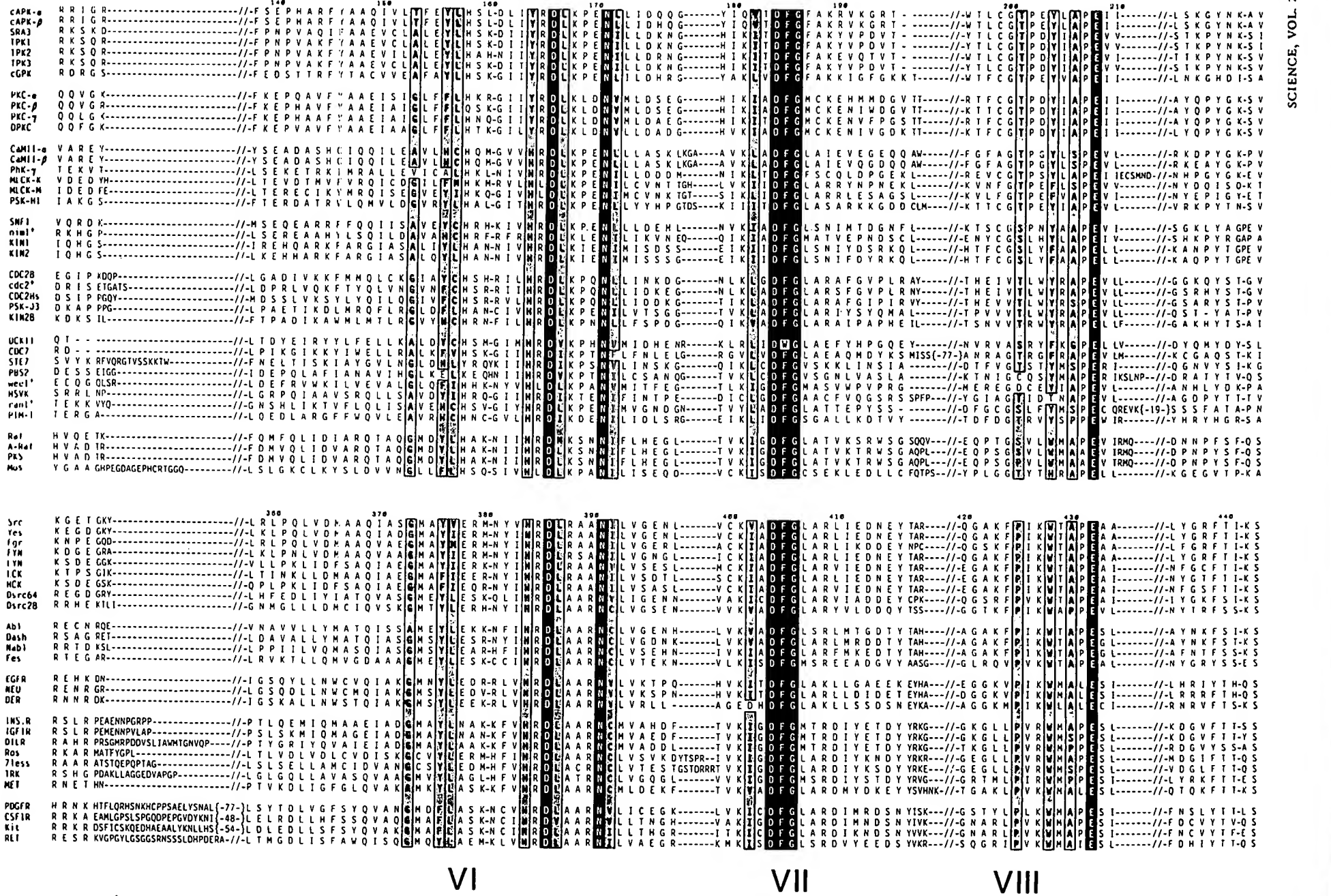
The next most populous cluster in the tree includes two separate subfamilies that can be classified according to their mode of regulation: the cyclic nucleotide–dependent protein kinases and the calcium-phospholipid–dependent protein kinases. The similarities in the mode of regulation of the members of these two subfamilies, namely, activation by "second messengers" released in response to ligand binding at the cell surface, may be a reflection of their recent evolutionary divergence.

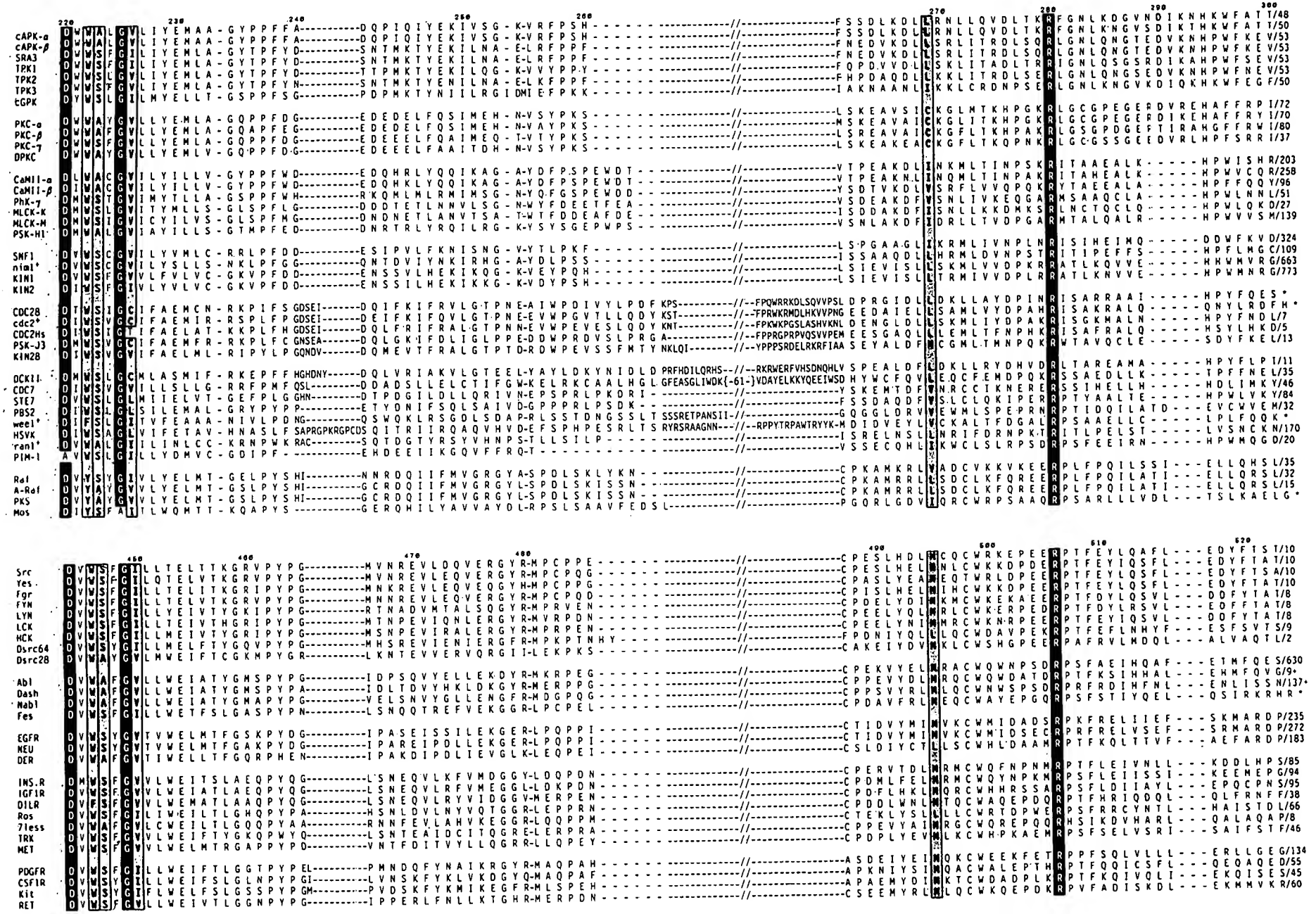
The third major catalytic domain cluster contains the subfamily of protein kinases that have activities regulated by calmodulin. The calmodulin-dependent cluster falls near the cyclic nucleotide– and calcium-phospholipid–dependent cluster. All members of the calmodulin-dependent subfamily have a calmodulin binding domain, characterized by a high proportion of basic amino acid residues and having a propensity for formation of an amphiphilic α helix, residing outside the catalytic domain. (Note that the calmodulin binding domain sequences were not included in the phylogenetic analysis.) The different protein kinases thus far described as being regulated by calmodulin, therefore, appear to have diverged from a common ancestor after acquisition of the calmodulin binding domain. The mapping of the putative protein kinase PSK-H1 within this cluster predicts that this enzyme will also prove to be regulated by calmodulin.

Also mapping near the cyclic nucleotide– and calcium-phospholipid–dependent protein kinases is a small cluster composed of four protein kinases recently identified in the budding or fission yeasts; SNF1, $nim1^+$, KIN1, and KIN2. Whether these protein kinases

Fig. 1. Multiple amino acid sequence alignment of 65 protein kinase catalytic domains. The first 38 sequences derive from protein–serine/threonine kinases (indicated by asterisks in Table 1) and the remaining 27 sequences in the alignment are from protein-tyrosine kinases (indicated by asterisks in Table 2). cAPK-α and Src have been chosen as prototype protein–serine/threonine and protein-tyrosine kinases, respectively; their catalytic domain sequences are numbered to indicate residue position from the polypeptide amino terminus. (Although the human Src sequence is shown, the numbering is actually taken from the chicken Src sequence to maintain established convention). The number of additional amino- and carboxyl-terminal flanking residues lying outside the catalytic domains are shown at the beginning and end, respectively, of each sequence. In several cases the sequences have not been determined through to the polypeptide amino or carboxyl termini; for these, the number of determined residues is given followed by a plus (+) sign. An asterisk (*) at the beginning or end of a sequence indicates that no additional flanking residues are contained in the polypeptide. Gaps, represented by dashes, were introduced into the sequences to optimize the alignment. In six cases, long insert segments have been excluded from the alignment to shorten the figure. The positions and lengths of the excluded inserts within the alignment are indicated by numbers within braces (for example, {-48-}); the excluded gap positions in the other sequences that correspond to these long inserts are shown as double slashes (//). Residues conserved in 62 or more of the 65 sequences are shown as white letters in black boxes. Positions where residues of similar structure are conserved in 63 or more sequences are shown in shaded boxes. Structurally similar groupings used for this purpose are nonpolar chain R groups (M, L, I, V, and C); aromatic or ring-containing R groups; (F, Y, W, and H); small R groups with near neutral polarity (A, G, S, T, and P); acidic and uncharged polar R groups (D, E, N, and Q); and basic polar R groups (K, R, and H). The single-letter amino acid code is used (A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine). Roman numerals at bottom indicate conserved subdomains.

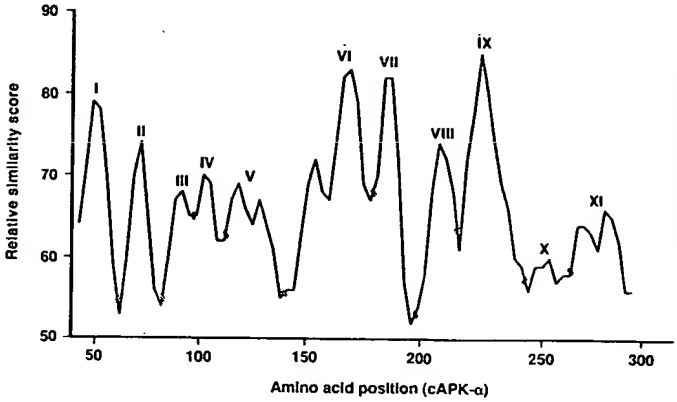

I
II
III
IV
V

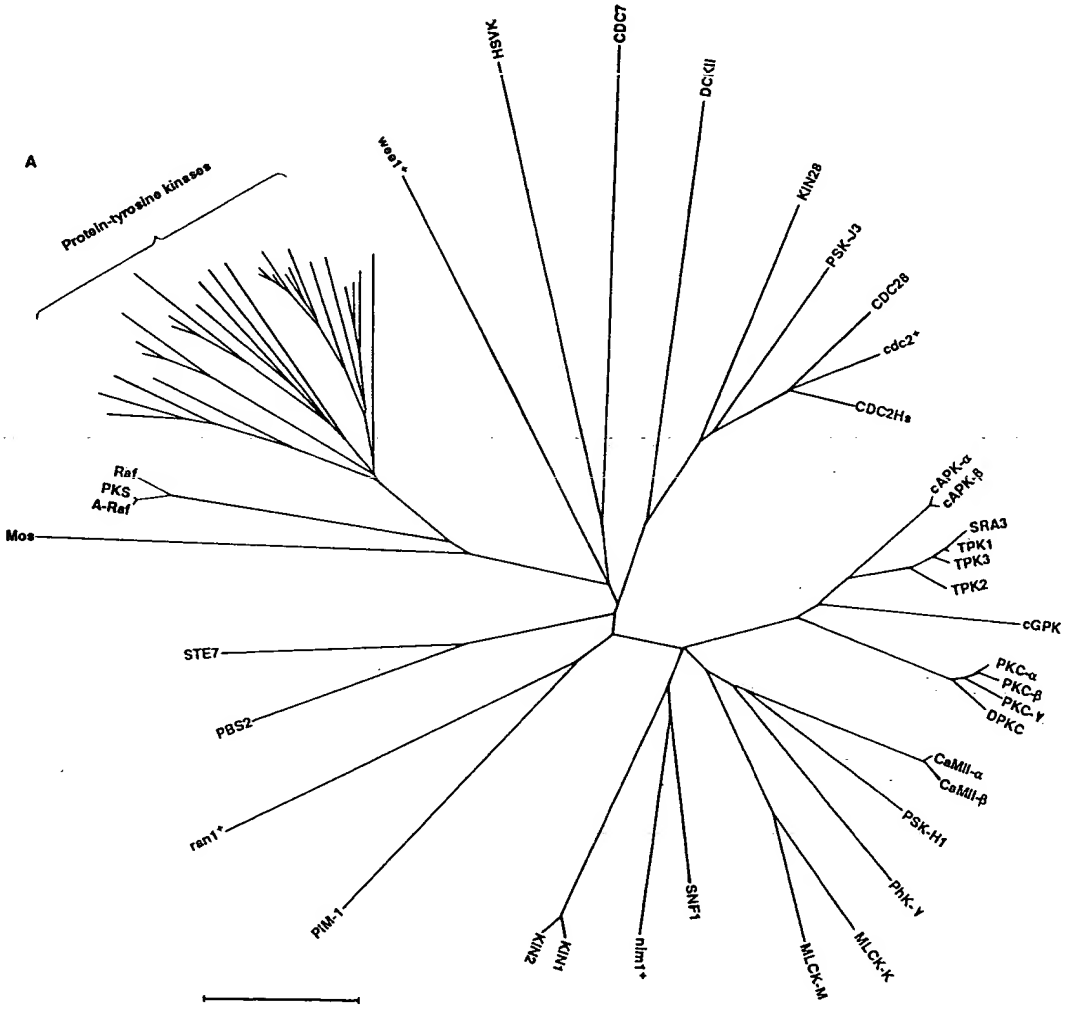

VI
VII
VIII



IX X XI

Fig. 2. Similarity profile of protein kinase catalytic domains. For each position in the alignment shown in Fig. 1, a relative similarity score was determined based on the "structure-genetic" scoring matrix (*116*) for amino acid similarities. Similarity scores were calculated as the sum of all possible pairwise comparisons between the individual amino acids at each position and expressed as the percentage of the highest possible score (that is, the score obtained when an identical residue occupies the position in all 65 aligned sequences). To smooth out the curve, a 9-position running average of the relative scores was determined, and every third position was plotted. Positions that contain gaps for ten or more of the sequences were not included in the profile; however, the locations of the major gap sites are indicated by breaks in the curve. The mean relative score for all the positions included in the profile is 66 with a standard deviation of 14.9. Relative similarity scores obtained when the catalytic domain sequences were randomly scrambled had a mean of 47 and standard deviation of 1.85. Roman numerals indicate conserved subdomains.





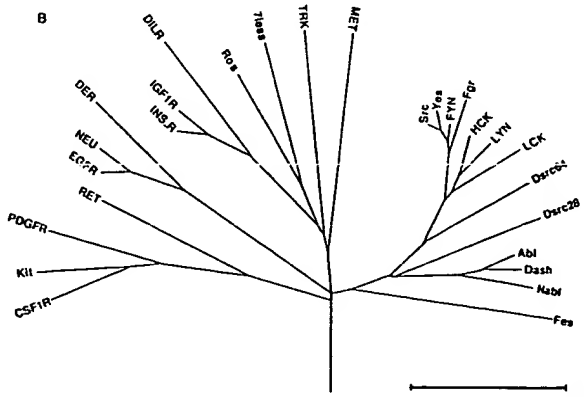


Fig. 3. Deduced phylogeny of protein kinase catalytic domains. The phylogenetic tree was constructed from the multiple alignment shown in Fig. 1. The tree-building concept of Fitch and Margoliash (*117*) was used as implemented by Feng and Doolittle (*118*). Briefly, similarity scores were obtained for all possible pairwise comparisons and transformed into a difference matrix from which branch order and length were determined. Programs were run on a VAX-785 computer equipped with 40 megabytes physical memory under virtual memory operating system (VMS). Systems limitations required that the branch lengths for the protein–serine/threonine and protein-tyrosine kinases be calculated separately, and the tree shown is thus a composite of these two determinations. The position of the protein-tyrosine kinase cluster was determined by including two protein-tyrosine kinases (Src and EGFR) in the protein–serine/threonine kinase tree construction. The individual sequences are indicated by the abbreviated names in Tables 1 and 2. The protein-tyrosine kinases are not labeled in (**A**), but are shown in the cluster enlargement in (**B**). The tree is shown "unrooted" in (A) as the branches are all measured relative to one another with no outside reference point. The scale bars represent a branch length corresponding to a relative difference score of 25. The tree depicted is likely to underestimate distances between the least related members of the family, particularly since the alignment used in its construction is parsimonious.

have similar modes of regulation remains to be determined. KIN1 and KIN2 were identified through screening a *Saccharomyces cerevisiae* DNA library with probes designed to recognize sequences characteristic of protein-tyrosine kinases and, as such, have been suggested to represent "structural mosaics" with some features of catalytic domain structure more indicative of the protein-tyrosine kinases than the protein–serine/threonine kinases (*5*). The deduced phylogeny of KIN1 and KIN2, however, does not suggest a close evolutionary relationship with protein-tyrosine kinases. In fact, the probe target used to identify KIN1 and KIN2 encodes the stretch of amino acids corresponding to cAPK-α Asp^{220}-Gly^{225} in conserved subdomain IX, a region of high conservation in all of the catalytic domains regardless of substrate specificity.

The subfamily related to CDC28 includes functional homologs from three widely divergent species: CDC28 from the budding yeast *S. cerevisiae*, $cdc2^+$ from the fission yeast *Schizosaccharomyces pombe*, and human CDC2Hs. Functional homology was demonstrated by heterologous complementation of conditional mutants defective in cell cycle progression (*32, 33*). The other two sequences mapping within this cluster are putative protein kinases identified in *Saccharomyces cerevisiae* (KIN28) and human HeLa cells (PSK-J3). The members of this cluster are also distinguished by the small sizes of the catalytic domain-containing polypeptides, suggesting their activities may be regulated through association with other polypeptides in a holoenzyme complex. Indeed, support for this notion has been obtained for $cdc2^+$ (*34*).

Perspectives

The tremendous diversity of the protein kinase family is just now beginning to be appreciated. Most of the catalytic domain sequences referenced in Tables 1 and 2 were reported within the past 2 years. With continued characterizations of regulatory mutants in invertebrates, along with the recent development of new hybridization approaches for the identification of DNA clones that encode novel protein kinase catalytic domains, it is likely that the rate of discovery will continue to accelerate through the next several years. The difficult tasks will be to confirm protein kinase activities for the newly identified family members and to elucidate their functional roles. Clues to function may come through an analysis of catalytic domain primary structure and subsequent phylogenetic mapping. A catalytic domain that has only limited divergence from another, better characterized, member of the family can be expected to play a similar role in cellular physiology. Further clues are likely to come from an inspection of amino acid sequences lying outside the catalytic domain where residues involved in enzyme regulation may be found.

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